RIBULOSE-1.5-BISPHOSPHATE CARBOXYLASE-OXYGENASE AND CARBON DIOXIDE FIXATION IN THE *RHODOSPIRILLACEAE*

by

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This thesis is presented for the
Degree of Doctor of Philosophy
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July 1985

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Acknowledgements

I should like to thank my supervisor, Dr. C. S. Dow, for his enthusiastic help, guidance and encouragement throughout the course of this project, and also to Dr. Lubert Dijkhuizen, University of Groningen, The Netherlands for supervising the continuous culture work.

I wish to thank all members of the Microbiology II Group for their friendliness and for providing a very pleasant working atmosphere in the laboratory.

I would like to acknowledge the financial support of the University of Ilorin, Nigeria and the Committee for Overseas Research Students (U.K.) for the studentship award.

Many thanks to Miss Kay Pollitt for typing this thesis.

Finally, I wish to thank my family for their continual support.

Declaration

I declare that this thesis has been composed by myself, and has not been used in any previous application for a degree. The results presented here were obtained by myself under the supervision of Dr. C. S. Dow. The work in Section 3.6.2 was performed in collaboration with Dr. L. Dijkhuizen and Dr. C. S. Dow. All other sources of information have been acknowledged by means of reference.

Abbreviations

A	Absorbance
AMP	Adenosine monophosphate
ADP	Adenosine diphosphate
ATP	Adenosine triphosphate
Ci	Curie
DMSO	Dimethylsulphoxide
EDTA	Ethylenediamine tetra-acetic acid
g	gramme
xg	gravitational force
nm	nanometers
PMSF	Phenylmethylsulfonyl fluoride
PPO	2,5-diphenyloxazole
SDS	Sodium dodecyl sulphate
TCA	Trichloroacetic acid
v/v	volume per volume
w/v	weight per volume

Summary

A single step purification procedure has been developed for the isolation of ribulose bisphosphate carboxylase/oxygenase from a number of photosynthetic bacteria. The method involves centrifugation of the soluble protein extracts on step sucrose gradients.

Two different molecular forms of ribulose bisphosphate carboxylase/oxygenase have been purified and characterised from Rhodopseudomonas blastica. The enzymes both have carboxylase and oxygenase activities. One of the enzymes (designated Form I) structurally resembles the plant enzymes, having eight large and eight small subunits, whereas the other (Form II) is composed of six large subunits and lacks small subunits. Peptide mapping of the isolated large subunits shows that the proteins are quite distinct, and are probably coded for by different genes.

The two enzymes show marked differences in kinetic properties. The Form I enzyme exhibits optimal activity at pH 8.0, is inhibited by low concentrations of 6-phosphogluconate and has a high affinity for CO_2 (Km CO_2 = 40 μM), while the Form II has a pH optimum of 7.4, is relatively insensitive to inhibition by 6-phosphogluconate and has a lower affinity for CO_2 (Km CO_2 = 102 μM).

The in vivo regulation of CO_2 fixation and synthesis of the Form I and the Form II enzymes in R. blastica has been studied using batch and continuous culture techniques. Evidence suggests that the synthesis of the Form I enzyme is subject to regulation by a repression/derepression mechanism and this is mediated mainly by the CO_2 concentration in the growth medium. The Form II enzyme when synthesised, is present at low levels and is subject to repression/derepression control by either energy or carbon limitations. The differences in the kinetic and regulatory properties suggest that Rhodopseudomonas blastica synthesises two different molecular forms of ribulose bisphosphate carboxylase/oxygenase as a means of physiological adaptation to variations in the levels of CO_2 in its growth environment.

Chapter 1 - General Introduction

1.1 The Definition of autotrophy

In recent years, the definition of autotrophy has received considerable attention. Early definitions were all on a nutritional basis, where autotrophs were defined as "organisms which are able to grow on inorganic nutrients" (Woods and Lascelles, 1954). Later definitions emphasised the inorganic nature of the energy source, e.g. light, or oxidation of inorganic compounds, as well as the inorganic carbon source. When the ribulose bisphosphate pathway (RuBP pathway) was discovered and it was found that many of the organisms referred to as autotrophs use this pathway for CO_2 assimilation, the operation of this pathway became synonymous with autotrophy, thereby changing from a nutritional to a metabolic basis for the definition (Kelly, 1971).

However, there are problems associated with the defining autotrophy when the designation is on a metabolic rather than a nutritional basis. For instance, several organisms such as Methylococcus capsulatus (Bath) (Taylor, Dalton and Dow, 1981) may use more than one pathway for carbon metabolism. Secondly, there are photosynthetic bacteria which possibly, have no ribulose bisphosphate pathway (Evans et al., 1966). Thirdly, the metabolism of many organisms has not been sufficiently elucidated to define the nature of the assimilatory pathway. In order to minimize these problems, the proposal by Whittenbury and Kelly (1977) to return to a nutritional basis for designating autotrophs is preferable. They defined autotrophs as "organisms which can synthesise all their cellular constituents from one or more one-carbon (C_1) compounds". This

definition broadens the scope of autotrophy to include those bacteria characterized as methylotrophs (Colby and Zatman, 1972).

For the purpose of clarity of presentation, the nutritional definition will be adhered to throughout this thesis and metabolic types will be indicated by naming the assimilatory pathway. In this regard, autotrophs are considered as those organisms able to grow on CO_2 as their sole carbon source, and methylotrophs are those able to grow on reduced carbon compounds containing one or more carbon atoms but no carbon-carbon bonds.

Members of the Rhodospirillaceae (formerly the non-sulphur photosynthetic bacteria) have been studied in this work with a view to understanding the role played by CO_2 in their metabolism. Therefore, the present knowledge of assimilation of C_1 compounds, particularly carbon dioxide, by microorganisms will be reviewed. Carbon dioxide fixation by higher plants will be discussed where relevant.

1.2 Assimilation of one carbon compounds

The diversity of microorganisms able to grow on one-carbon compounds as the sole source of carbon and energy (see below) is impressive, but even more remarkable is the diversity of their assimilatory pathways. Four different pathways have been proposed by which autotrophs and methylotrophs assimilate carbon substrates into cell material. These are: a) the ribulose bisphosphate pathway or Calvin Cycle of CO_2 assimilation, b) the reductive carboxylic acid cycle for CO_2 fixation, c) the Ribulose monophosphate cycle of formaldehyde fixation, and d) the serine pathway. The latter two pathways are used mainly by the

methylotrophs for the net assimilation of reduced one-carbon compounds. These pathways and their proposed variants will now be considered.

1.2.1 Ribulose bisphosphate pathway (Calvin Cycle) of CO_2 fixation

1.2.1.1 Elucidation of the Calvin Cycle

The primary route by which inorganic CO_2 is assimilated into utilizeable organic matter in autotrophic organisms is by the reactions of the Calvin-Benson reductive pentose phosphate pathway (Ribulose bisphosphate pathway). Calvin and co-workers first elucidated the pathway in the green algae, Chlorella (Calvin, 1962). The techniques used by Calvin and co-workers have become the basis for the most work using radioisotopes for tracing metabolic pathways and this will be considered briefly.

Calvin developed two main techniques for tracing metabolic pathways: a) rapid sampling following fixation of [^{14}C]-bicarbonate and the identification of labelled compounds; b) carbon-14 saturation experiments. In this work, a suspension of algae undergoing photosynthesis under atmospheric CO_2 were injected with a stream of ^{14}C-labelled CO_2/bicarbonate for a suitable length of time and the organisms killed. The amount of radioactivity (radioisotope) present in various cellular compounds were measured by plotting the radioactivity against time. Curves of varying slopes and shapes were obtained. Curves for compounds serving as ports of entry had negative slopes and those compounds derived from them, positive slopes. Using this technique, Calvin and co-workers were able to identify several intermediates of the Calvin Cycle and also the product of carboxylation-phosphoglyceric acid. In addition, Calvin and co-workers recognised that most of the compounds identified, apart from sucrose, became saturated with radioactivity very

quickly, and yet the amount of these compounds present at any time was small and did not change. The radioactivity saturation levels for the compound through which carbon was flowing was used to measure the total amount of active pool size of these compounds in Chlorella. By changing external variables, alteration in pool sizes could be followed.

Calvin found that when photosynthesizing cells of Chlorella were deprived of light, there was an immediate and sudden rise in the level of phosphoglycerate accompanied by a corresponding fall in the level of ribulose bisphosphate (RuBP). It was then concluded that the removal of light blocked the easy conversion of phosphoglycerate (PGA) to triose phosphates without any decrease in the rate of formation of PGA from RuBP. As RuBP cannot be produced in the dark, this suggested that PGA was derived from RuBP. Wilson and Calvin (1955) initially showed that when growing cultures of Chlorella were limited for CO_2 in the presence of light, there was an accumulation of RuBP and a fall in the level of the PGA pool.

The results of these labelling experiments were put together with the studies of enzyme activities in cell-free extracts to elucidate the cycle of carbon dioxide fixation known as the Calvin-Benson Cycle (Ribulose bisphosphate pathway). The overall scheme of the Calvin Cycle is shown in Figure 1.1 and an alternative sequence of rearrangement to the basic scheme is shown in Figure 1.2.

1.2.1.2 The Reactions of the Calvin Cycle

The basic cycle of Calvin (Ribulose bisphosphate pathway) is represented in Figures 1.1 and 1.2. The first key step in this cycle is the carboxylation of RuBP to give 3-phosphoglycerate the fixation step. This

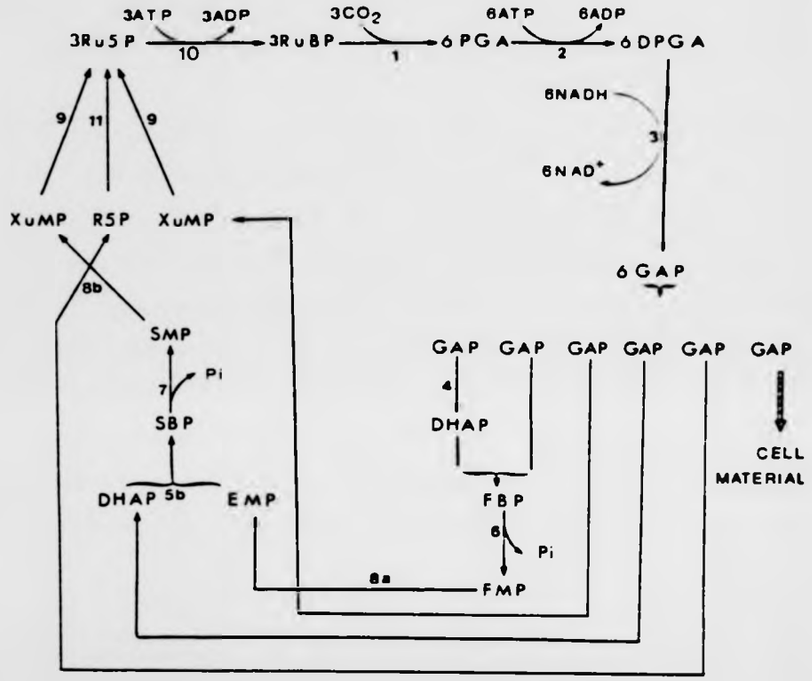


Figure 1.1 The Ribulose Bisphosphate Cycle (Calvin Cycle) of CO_2 fixation (sedoheptulose bisphosphate variant)

Abbreviations: RuBP: ribulose-1,5-bisphosphate; PGA: 3-phosphoglyceric acid; DPGA: 1,3-diphosphoglyceric acid; GAP: glyceraldehyde 3-phosphate; FBP: fructose-1,6-bisphosphate; FMP: fructose-6-phosphate; EMP: erythrose-4-phosphate; SBP: sedoheptulose 1,7-bisphosphate; SMP: sedoheptulose-7-phosphate; XuMP: xylulose-5-phosphate; R5P: ribose-5-phosphate; Ru5P: ribulose-5-phosphate.

The enzyme numbers referred to in the text are:

1) ribulose bisphosphate carboxylase-oxygenase
2) phosphoglycerate kinase
3) glyceraldehyde phosphate dehydrogenase
4) triose phosphate isomerase
5a,b) aldolase
6) fructose bisphosphatase
7) sedoheptulose biphosphatase
8) transketolase
9) pentose phosphate epimerase
10) phosphoribulokinase
11) pentose phosphate isomerase

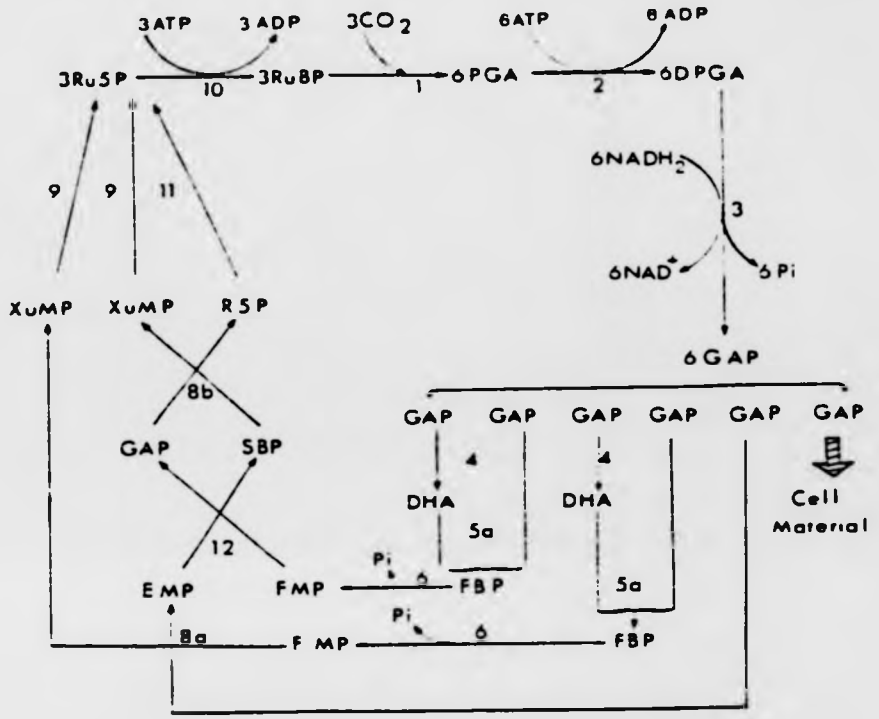
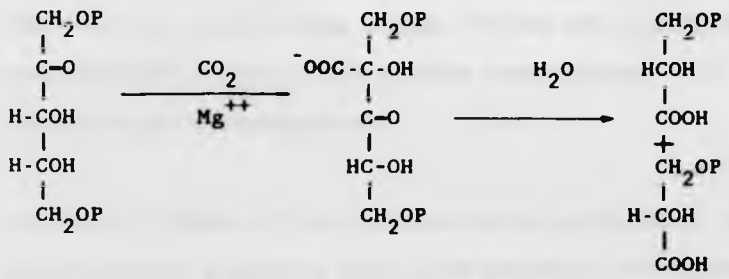


Figure 1.2 The Ribulose bisphosphate cycle of CO_2 assimilation (transaldolase variant)

Abbreviations and enzymes are as in Figure 1.1 except (12) transaldolase.

reaction is catalysed by a Mg^{2+} requiring enzyme, ribulose bisphosphate carboxylase/oxygenase (RuBisCO) (1):

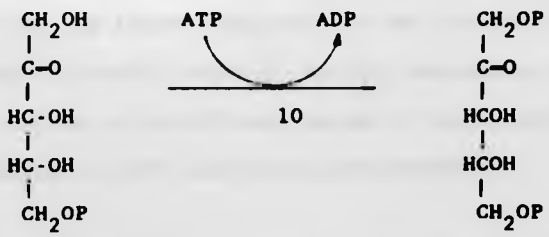


RuBP 2,PGA

The product of this reaction, phosphoglyceric acid, is then phosphorylated to give 1,3-diphosphoglycerate (DPGA).

The second part of the cycle is the reduction of the DPGA to glyceraldehyde phosphate (GAP) in a reaction requiring reduced nucleotides.

The remainder of the cycle functions to regenerate the CO_2 acceptor molecule, ribulose bisphosphate (RuBP). This involves a series of rearrangement sequences of which the final step is catalysed by phosphoribulokinase - the second key enzyme of the Calvin Cycle. In this reaction, phosphoribulokinase catalyses the phosphorylation of Ribulose monophosphate (RuMP) to form RuBP - an irreversible reaction requiring ATP (10).



Ribulose-5-phosphate Ribulose bisphosphate

At least two variants of this cycle which differ only in the reactions involved in rearrangement are known. The sedoheptulose bisphosphatase (SBP) variant involve this enzyme (7) but not transaldolase, whereas the transaldolase variant (TAL) involve transaldolase (12) but not sedoheptulose bisphosphatase.

The overall scheme of the reaction cycle synthesises one molecule of glyceraldehyde phosphate from three molecules of carbon dioxide, six moles of $NAD(P)H_2$ and nine moles of ATP - indicating the high energy consuming nature of the cycle:

$$3CO_2 + 6NAD(P)\,H + 6H^+ + 9ATP \longrightarrow \text{glyceraldehyde-3-phosphate} + 6NAD(P) + 9ADP + 8Pi$$

The presence of RuBP carboxylase/oxygenase and phosphoribulokinase in cell-free extracts is generally taken as an indication of the operation of the Calvin Cycle.

Before considering the occurrence and regulation of the Ribulose bisphosphate (RuBP) pathway (Calvin Cycle) mention will be made of a second pathway of CO_2 fixation in certain higher plants which was proposed by Hatch and Slack (1966). This was based on their finding that after one second of exposure of detached leaf segments to $^{14}CO_2$, more than 93% of the fixed radioactivity was located in carbon-4 of dicarboxylic acids, notably, malate, aspartate and oxaloacetate. After longer periods, a significant amount of radioactivity was located in 3-phosphoglyceric acid and hexose monophosphate.

The scheme proposed to account for these observations is shown in Figure

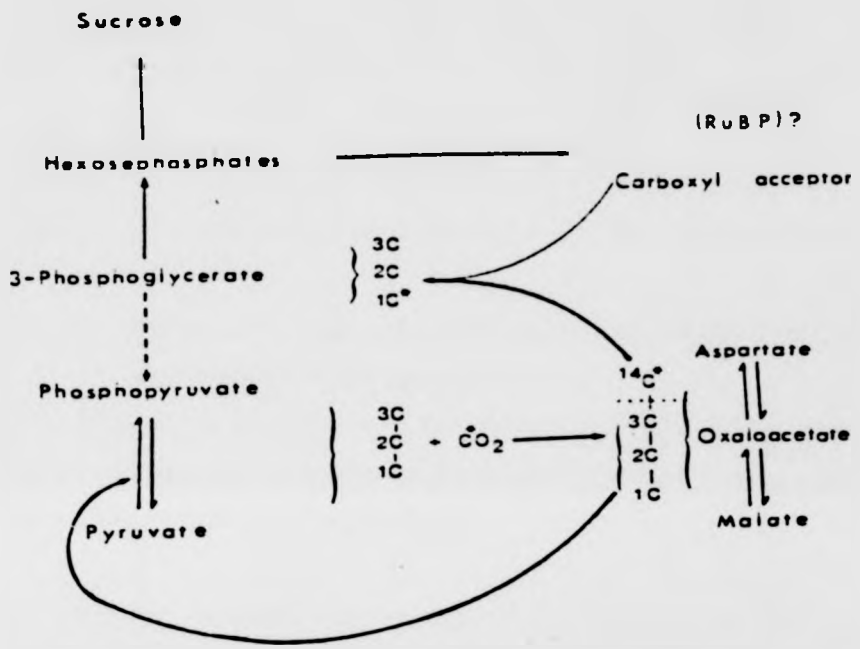


Figure 1.3 Proposed pathway for photosynthetic fixation of CO_2 in C_4-plants.

The broken arrow indicates a minor pathway.

(After Hatch and Slack, 1966).

1.3. In this pathway, CO_2 is first incorporated into the C_4 dicarboxylic acids pool and then transferred to sugars via 3-phosphoglycerate. Plants in which this pathway occurs are thought to exhibit a higher rate of photosynthesis than the C_3-plants, which have 3-phosphoglycerate as the primary fixation product. However, it should be noted that Zelitch (1975) has pointed out that carboxylation yielding malate and aspartate represents a mechanism for increasing the concentration of CO_2 at the site of action of RuBP carboxylase and not a net fixation pathway.

1.2.1.3 The occurrence and distribution of the Calvin Cycle (Ribulose bisphosphate (RuBP) pathway)

To establish the occurrence of the RuBP pathway in microorganisms with the same degree of thoroughness as in green plants, the following requirements had to be satisfied:

a) The first labelled compound during assimilation of $^{14}CO_2$, must be phosphoglyceric acid. Other phosphorylated intermediates must also be labelled at early times.

b) All the enzymes necessary for the operation of the cycle must be present in the organism. In practice, however, the presence of RuBisCO (carboxymutase) and phosphoribulokinase in cell-free extracts is generally taken as an indication of the operation of the pathway.

Most of the work on CO_2 fixation in prokaryotes has been done with the photosynthetic bacteria, notably the Rhodospirillaceae. These organisms, although able to grow photoautotrophically, exhibit higher growth rates under heterotrophic conditions where CO_2 fixation assumes less importance. Among the photosynthetic bacteria, Rhodospirillum rubrum which may be

grown photoautotrophically using CO_2 and H_2 as carbon and energy source respectively, and photoheterotrophically, has been the subject of several investigations into the mechanism of CO_2 fixation. Glover et al. (1952) first showed that 3-phosphoglycerate was the first major product of CO_2 fixation in photoautotrophically grown cells of R. rubrum. Hoare (1963) obtain similar results when R. rubrum was grown on malate. Anderson and Fuller (1967a) showed that after one second of incubation, autotrophically grown cells of R. rubrum incorporates more than 75% of the radioactivity of $^{14}CO_2$ into 3-phosphoglyceric acid and phosphate esters. These results clearly indicate that under photoautorophic conditions, the Calvin cycle is the major pathway of CO_2 assimilation in this organism. Growth conditions were also found to have a profound effect on the pattern of uptake of $^{14}CO_2$. Porter and Merrett (1972) and Slater and Morris (1973a) have shown that light intensity influences the uptake of CO_2 and RuBisCO activity in R. rubrum. with both parameters having higher activities in cells grown at higher light intensities.

Kinetic studies with Rhodopseudomonas palustris (Stoppani et al., 1955) showed that CO_2 is fixed via the Calvin Cycle. However, contradictory results have been published by Yoch and Linderstrom (1967) using Rhodopseudomonas palustris grown on CO_2 or formate. They reported that R. palustris photoassimilated each substrate into amino acids in the shortest fixation time of 15 seconds. Moreover, they also showed that very little of the total radioactivity could be found in any of the phosphate esters. These results were suggestive of a reductive carboxylic acid cycle. In contrast to these results, Stokes and Hoare (1969), showed that photoautotrophically grown cells of R. palustris utilising ^{14}C-formate and bicarbonate, produced phosphate esters as the first assimilation products and that the key enzymes of the Calvin Cycle

were present in cell-free extracts. The majority of results thus far obtained, indicate that the Calvin Cycle is the major assimilatory pathway during growth of most photosynthetic prokaryotes utilizing CO_2 as the main or sole carbon source.

In the oxygenic photosynthetic prokaryotes, i.e. the cyanobacteria, evidence from early $^{14}CO_2$ kinetic studies showed that the Calvin Cycle functioned in CO_2 assimilation in these organisms (Norris et al., 1955). Detailed studies of $^{14}CO_2$ fixation in the light and during subsequent dark periods after steady-state photosynthesis, were in complete accordance with the operation of the Calvin Cycle (Pelroy and Bassham, 1972). The RuBP pathway of CO_2 fixation has also been demonstrated in Halobacterium halobium, a photosynthetic halophilic bacterium (Danon and Kaplan, 1977).

In the non-photosynthetic bacteria, notably the chemosynthetic microorganisms, a number are able to grow on formate using the energy obtained from the oxidation of the substrate for the autotrophic assimilation of CO_2 via the Calvin Cycle (Bassham et al., 1954). Table 1.1 shows a number of chemosynthetic bacteria which assimilate CO_2 via the Calvin cycle during growth on C_1- compounds. It should be noted that Pseudomonas oxalaticus, an otherwise typical heterotroph, assimilates its carbon substrate by the Calvin cycle only when it is growing on formate as carbon and energy source but not on other C_1-compounds (Quayle and Keech, 1960).

The possibility of the Calvin Cycle operating in Methylococcus capsulatus (Bath) when growing on methane has been demonstrated by Taylor et al. (1981), although only about 2.5% of the cell carbon arose from CO_2 during

Table 1.1 Bacteria which assimilate CO_2 via the Calvin Cycle during Growth on C_1 compounds

Organism	Carbon Source	Reference
Pseudomonas oxalaticus (OX1)	formate	Quayle and Keech (1959) Dijkhuizen and Harder (1979a, b)
Thiobacillus A_2	formate	Kelly et al. (1979); Wood and Kelly (1981); Gottschal and Kuene (1981)
Thiobacillus novellus	formate	Chandra and Shethna (1976)
Alcaligenes eutrophus H-16	formate	Friedrich et al. (1979)
Alcaligenes FOR1	formate	Chandra and Shethna (1976)
Paracoccus denitrificans	methanol	Cox and Quayle (1975)
	formate	van Verseveld and Stouthamer (1978)
Achromobacter IL	methanol	Loginova and Trotsenko (1979)
Pseudomonas 8	methanol	Loginova and Trotsenko (1979)
Mycobacterium 50	methanol	Loginova and Trotsenko (1979)
Blastobacter viscosus	methanol	Loginova and Trotsenko (1979)
Bacterium formoxidans	formate	Sorokin (1961)
Hydrogenemonas eutropha Z-1	formate	Namasaraev et al. (1971)
Methylococcus capsulatus	methanol	Taylor et al. (1981)

growth on methane as carbon substrate. This is of interest, since it has long been noted that the predominant carbon assimilatory pathway in methanotrophs is by the Ribulose monophosphate pathway (Section 1.3.1). If the RuBP pathway of CO_2 fixation evolved from the ribulose monophosphate pathway of formaldehyde assimilation, as suggested by Quayle and Ferenci (1978), then M. capsulatus (Bath), having both assimilatory pathways, represent an important transition microbe.

The Calvin Cycle has also been demonstrated to be the major pathway of CO_2 fixation in certain hydrogen bacteria (Bergmann et al., 1958), and Pseudomonas facilis (McFadden, 1959). This cycle has also been implicated in the CO_2 metabolism of Desulphovibrio vulgaris, where it has been shown that cell-free extracts incorporate $^{14}CO_2$ into acid stable product via an RuBP dependent route(Alvarez and Barton, 1977).

In conclusion, the observations made with diverse organisms within which the Calvin Cycle operates, makes this cycle the only proven, indispensible mechanism of autotrophic CO_2 fixation.

1.2.1.4 Regulation of the Calvin Cycle

The reaction sequence of the RuBP pathway (Calvin Cycle) cons titutes the main pathway of CO_2 assimilation in eukaryotic and the vast majority of prokaryotic autotrophs. Production of biomass, therefore, depends largely on the operation of this primary biosynthetic route. The regulation of the Calvin Cycle would be expected to be of primary importance in higher plants and algae during light and dark transitions, and in many bacteria which exhibit heterotrophic and autotrophic metabolism. This section will deal mainly with the regulation of this cycle in bacteria, since the details on the regulation of photosynthetic

carbon metabolism in plants has been documented in the reviews by Kelly et al. (1976) and Walker (1976).

In bacteria, two major levels of control of the RuBP pathway are easily recognized, namely, control of enzyme activity and control of enzyme levels. These will be considered in turn.

1.2.1.4.1 Control of enzyme activity

a) By energy charge

Johnson and Peck (1965) first showed the inhibition by adenine monophosphate (AMP) of ribose-5-phosphate, ATP dependent carbon dioxide fixation in extracts of Thiobacillus thioparus. Similar effects by AMP were observed with cell-free preparations of Chromatium D (Johnson, 1966), Hydrogenomonas facilis (McFadden and Tu, 1967), T. novellus (Aleem and Huang, 1965) and Rhodopseudomonas sphaeroides (Rindt and Ohman, 1969). It has therefore been suggested that this phenomenon afforded a control mechanism by which ATP could be conserved in times of energy deprivation. In otherwords, the energy consuming Calvin Cycle is only allowed to function if sufficient energy is available (Johnson and Peck, 1965). Evidence thus far obtained suggested that one locus of inhibition by AMP is the step catalysed by phosphoribulokinase(McFadden and Tu, 1967; MacElroy et al., 1969; Abdelal and Schlegel, 1974a). However, it should be noted that in contrast to the marked inhibitory effects by AMP, it has been reported that partially purified phosphoribulokinase (PRK) from Thiobacillus neopolitanus was only slightly inhibited by 3.5 mM AMP (MacElroy et al., 1972).

Of interest is the finding of Charles and White (1976) that RuBisCO from Thiobacillus A_2 responds positively to energy charge and that this

response is decreased by 6-phosphogluconate, a ligand that is inhibitory under all conditions. The significance of 6-phosphogluconate as a regulator of Ribulose bisphosphate carboxylase/oxygenase (RuBisCO) is discussed in section (1.5.6). Other steps that are inhibited by AMP are those catalysed by 3-phosphogylcerate kinase, as found in Hydrogenomonas facilis (McFadden and Schuster, 1972), and fructose-1,6-biphosphatase in T. neopolitanus (Johnson and MacElroy, 1973). In the face of these reports, Atkinson (1970) has suggested that regulation by energy charge at one or more of these key steps in the biosynthetic Calvin Cycle is of importance in the fine control of this cycle in autotrophic bacteria.

b) By NADH

Specific activation by NADH is a typical property of the phosphoribulokinase from hydrogen oxidizing bacteria (Abdelal and Schlegel, 1974a; MacElroy et al., 1969; Siebert et al., 1981). Other reports have indicated that NADH is also a positive effector of phosphoribulokinase (PRK) in the nitrifier, Nitrobacter winogradskyi (Kiesow et al., 1977) and some members of the Rhodospirillaceae (Rindt and Ohman, 1969; Tabita, 1981; Bowien, 1983). The regulation of phosphoribulokinase by NADH and AMP is compatible with the high demand of CO_2 assimilation for reducing power and energy.

c) By other metabolites

The possibility of positive and negative control of the Calvin Cycle by the intermediates of intermediary metabolism was first reported by Ballard and MacElroy (1971), who showed that phosphoribulokinase (PRK) from H. facilis was strongly inhibited by phosphoenol pyruvate. A similar effect was reported by Hart and Gibson (1971), who found that the

presence of glyceraldehyde-3-phosphate at 0.04 mM resulted in inhibition of the Chromatium D enzyme.

Recently, Bowien (1983) demonstrated that the activity of PRK from cell-free extracts of a number of strains of Rhodospirillaceae is modulated by phosphoenolpyruvate. This modulation, by an indirect end product of the Calvin Cycle is therefore regarded as classical feedback inhibition of the first enzyme in a biosynthetic pathway. Interestingly, plant PRK is inhibited by 3-phosphoglycerate, the first stable product of the Calvin Cycle (Gardemann et al., 1982).

An important intermediate in the oxidative pentose phosphate pathway and the Entner-Doudoroff pathway is 6-phosphogluconate which is an inhibitor of RuBisCO. Inhibition of the Calvin Cycle by this metabolite provides a control mechanism between heterotrophic metabolism and reductive CO_2 fixation.

1.2.1.4.2 Control of Enzyme Synthesis

The control of synthesis of the Calvin Cycle enzymes particularly during transition from heterotrophic to autotrophic metabolism has been studied in recent years. It has been assumed that three enzymes function uniquely in the Calvin Cycle during the growth of autotrophic bacteria, namely ribulose bisphosphate carboxylase/oxygenase (RuBisCO), phospho-ribulokinase (PRK) and alkaline fructose-1,6-bisphosphatase(FBP) which may also function as sedoheptulose-1,7-bisphosphatase.

Control of the synthesis of the enzyme involved in the carboxylation reaction, i.e. RuBisCO is considered in detail in section 1.5.6. However, studies with several members of the Rhodospirillaceae have

revealed how rates of CO_2 fixation and the synthesis of the enzymes of the Calvin Cycle are regulated during both autotrophic and heterotrophic growth. The following conditions have been recognised: (i) during anaerobic photoautrophic growth on H_2 and CO_2, high levels of RuBisCO and PRK are paralleled by high rates of CO_2 fixation (Anderson and Fuller, 1967b; Slater and Morris, 1973a; Schloss et al., 1979; Tabita, 1981). Under these conditions, CO_2 is the sole source of carbon and consequently the Calvin Cycle provides the cellular carbon.

(ii) During anaerobic growth as photoheterotrophs, with organic carbon compounds serving as the electron donor and light as the energy source, RuBisCO and PRK are present at low (repressed) levels if the electron donor is an oxidized carbon compound, e.g. malate (Slater and Morris, 1973a; Tabita, 1981). On the other hand, if the electron donor is a reduced fatty acid, e.g. butyrate, both enzymes are derepressed and present at high levels (Tabita and McFadden, 1974a; Tabita, 1981).

(iii) Growing aerobically in the dark as chemoheterotrophs, Calvin Cycle enzymes are not produced.

(iv) When growing as fermentative anaerobes, in darkness, on sugars as the sole carbon and energy sources, the Calvin Cycle does not operate and the enzymes are repressed.

(v) During dark aerobic growth as chemo-autotrophs, with H_2 as the source of energy, oxygen as the electron acceptor, and CO_2 as the carbon source (Madigan and Guest, 1979) these microbes behave as typical hydrogen bacteria. In this growth mode, moderate levels of the Calvin Cycle enzymes are synthesised.

From this short discussion, it is obvious that the Rhodospirillaceae have developed very effective mechanisms for controlling the synthesis of the Calvin Cycle enzymes. Indeed, it is envisaged that the two key enzymes, RuBisCO and PRK, are under operonic control since they catalyse consecutive reactions in the pathway and have been shown to be co-ordinately expressed under various nutritional conditions (Abdelal and Schlegel, 1974b; Tabita, 1981). In contrast to this view, McFadden and Tu (1967), McCarthy and Charles (1974) have suggested that the synthesis of RuBisCO and PRK are not co-ordinately regulated. This has been demonstrated in Hydrogenomonas facilis (McFadden and Tu, 1967) and in T. novellus (McCarthy and Charles, 1974).

Many facultative autotrophs show strong responses to changing nutritional conditions. In accord with the metabolic needs, the activities of the PRK and RuBisCO are generally much lower during heterotrophic growth (Bowien and Schleger, 1981). In Alcaligenes eutrophus, complete repression of both the RuBisCO and the PRK during heterotrophic growth on pyruvate, succinate, or acetate has been reported. Apparently, these two enzymes may be co-ordinately regulated (Friedrich et al., 1981; Leadbeater et al., 1982; Bowien, 1983).

Regulation of the Calvin Cycle enzymes in Pseudomonas oxalaticus is of particular interest. This organism can grow either on formate or oxalate as the sole source of carbon and energy; in both cases energy is derived from formate oxidation, oxalate being converted to formate through co-enzyme A thioesters. In cells grown on formate, the Calvin Cycle operates as the pathway utilised for carbon-metabolism, but when grown on oxalate, carbon is assimilated heterotrophically with the result that the Calvin

cycle enzymes are highly repressed (Quayle, 1961; Dijkhuizen et al., 1977). Blackmore and Quayle (1968) studying the effect of mixed carbon substrates on the expression of enzyme levels in P. oxalaticus, suggested that formate was an inducer of the Calvin cycle enzymes. However, the extended studies of Harder and his co-workers (Dijkhuizen et al., 1978; Dijkhuizen and Harder, 1979a) using substrate transition experiments and growth of P. oxalaticus in chemostats have suggested that the synthesis of the Calvin Cycle enzymes involve repression/derepression rather than induction. In fact, it has been shown by Dijkhuizen and Harder (1979a) that during growth on P. oxalaticus on oxalate in a chemostat at varying dilution rates, enzymes of the Calvin Cycle and autotrophic CO_2 fixation were derepressed. They attributed this observation to the decreasing concentration of intracellular repressor molecule(s), paralleled to the decreasing concentration of the growth-limiting substrates in the culture at the low growth rates (low dilution rates). However, to date there is no proof that the structural genes for RuBisCO and PRK, the key enzymes of the Calvin Cycle, are contigous or that they show one or more regulatory elements. These questions will have to be approached from a genetical standpoint to be answered.

1.2.1.4.3 Regulation by other mechanisms

RuBisCO containing polyhedral bodies termed carboxysomes occur in several types of carbon dioxide fixing microorganisms (Codd and Marsden, 1984). The presence of this enzyme in carboxysomes of autotrophic bacteria raises questions concerning the role these inclusion bodies have in relation to autotrophy. It has been suggested that these bodies may play a role as the active sites for CO_2 fixation, protect RuBisCO from adverse effects such as inhibition by oxygen, and protect from degradation by proteases. They may also act as CO_2-concentrating compartments for

RuBisCO or as general protein storage bodies. Evidence for and against these various roles has been presented in the recent review by Codd and Marsden (1984). The possibility that the entire Calvin Cycle may operate within the confines of the carboxysomes of some CO_2 limited cultures of T. neopolitanus has been raised (Beudeker et al., 1980). In view of their widespread occurrence amongst autotrophs (Codd and Marsden, 1984), they may well have a significant role in the regulation of the Calvin cycle. However, further knowledge of carboxysome composition, particularly structure and properties, in relation to autotrophic growth may go a long way to establishing their regulatory role in the Calvin cycle.

The possibility of light mediated regulation of the Calvin Cycle has been raised in recent years as in many flowering plants, the accumulation of RuBisCO is found to be stimulated by light (Gallagher and Ellis, 1982). The primary effect of light is thought to be mediated via phytochrome by a stimulation of transcription in both nuclear and chloroplast compartments (Gallagher and Ellis, 1982; Jenkins et al., 1983). Direct effects of light on the activation of the RuBisCO enzyme from photosynthetic eukaryotes have been shown by Dalley et al. (1978). The exact mechanism by which this is brought about is not yet known, and the physiological implication of these findings awaits further investigations.

1.2.2 The Reductive Carboxylic and Cycle for CO_2 fixation

In 1966, Evans and co-workers (Evans et al., 1966) first proposed a ferredoxin-dependent cycle of CO_2 fixation, termed the reductive carboxylic acid cycle. The cycle was first elucidated in the green bacterium Chlorobium thiosulfatophilum grown anaerobically in the light

with CO_2 as the sole source of carbon.

This cycle involves several enzymes of the tricarboxylic acid cycle, in addition to two novel carboxylation reactions catalysed by pyruvate and α-ketoglutarate synthetases, both of which required reduced ferredoxin. These two novel enzymes have been found in the cell-free extracts of Rhodospirillum rubrum (Buchanan et al., 1967), Chromatium D (Buchanan and Arnon, 1965), Methanobacterium thermoautotrophicum (Zeikus et al., 1977) and recently in Chlorobium limicola (Fuchs et al., 1980a). The overall scheme of the operation of the reductive carboxylic acid cycle is shown in Figure 1.4. One turn of the cycle results in the incorporation of four molecules of CO_2, the synthesis of one molecule of oxaloacetate, and regeneration of acetyl coA, the primary CO_2 acceptor.

The evidence for the operation of this cycle came mainly from the studies with C. thiosulfatophilum based upon the rapid incorporation of $^{14}CO_2$ into amino acids, notably, glutamate. However, the reviews by Quayle (1972) and McFadden (1973) have cast some considerable doubts on the evidence for the operation of this cycle in the organisms in which it has been reported to function. For instance, kinetic studies of Evans et al. (1966) and Sirevag and Ormerod (1970) did not include very early fixation times. Moreover, $^{14}CO_2$ fixation experiments were carried out at elevated physiological CO_2 concentrations. Furthermore, the extended studies of Buchanan et al. (1972) on CO_2 assimilation by C. thiosulfatophilum (Tassajara) showed that phosphoglycerate was the principal labelled product of $^{14}CO_2$ fixation after 5 seconds incubation, although they could not detect ribulosebisphosphate carboxylase/oxygenase (RuBisCO) in the extracts. However, RuBisCO has now been purified from C. thiosulfatophilum (Tasajara) Tabita et al. (1974a) although at a low

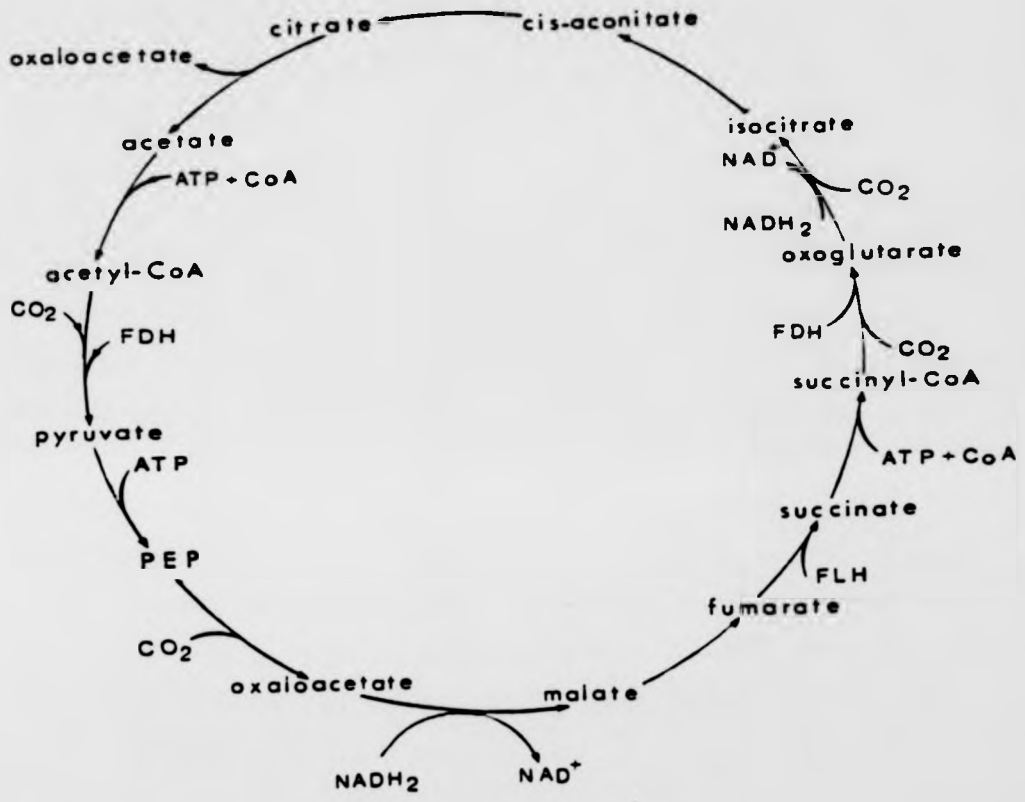


Figure 1.4 Reductive carboxylic acid cycle for CO_2 fixation

(After Evans et al., 1966).

specific activity. In addition, several enzymes, notably citrate lyase, have activity far too low in cell-free extracts to account for *in vivo* CO_2 fixation rate. Fuchs *et al*. (1980b) have suggested that the inability to detect, or the very low activity of, citrate lyase in *C. thiosulfatophilum* could be due to instability of the enzyme or to its regulatory properties.

In the face of the kinetic results, lower CO_2 concentrations and the detection of RuBisCO in some of the organisms believed to fix CO_2 by the reductive carboxylic acid cycle, it seems more likely that in these organisms, CO_2 is primarily fixed via the Calvin cycle. In conclusion, there is yet no substantive evidence for the reductive carboxylic acid cycle functioning as a major CO_2 fixing pathway during strictly autotrophic growth. Nor is there any compelling evidence for the functioning of the complete cycle during heterotrophic growth. However, certain bacteria may have the enzymic capability to support part of the cycle which in *M. thermoautotrophicum* (Fuchs *et al*., 1978; Fuchs and Stupperich, 1978) and *Chlorobium limicola* (Fuchs *et al*., 1980a, b) may have an important role.

1.3 Assimilation of Reduced One-Carbon Compounds

At the present time two pathways for the assimilation of reduced one-carbon (C_1) compounds are recognised. These are the ribulose monophosphate (RuMP) cycle and the serine pathway, both of which are involved in the assimilation of formaldehyde in methylotrophs. Several reviews concerning the elucidation and the operation of these pathways have been presented in recent years (Anthony, 1975; Colby *et al*., 1979).

Consequently, only a brief discussion of these pathways will be given here.

1.3.1 The Ribulose monophosphate cycle (RuMP pathway)

The RuMP pathway of formaldehyde assimilation was first proposed by Kemp and Quayle (1967). The work leading to the elucidation of this cycle was largely carried out with obligate methane and methanol oxidizers.

The operation of the cycle is very similar to the Calvin cycle, and occurs in three phases. The first phase (the fixation phase) involves the reactions unique to the pathway, namely the condensation of formaldehyde with ribulose monophosphate (RuMP) and the subsequent isomerization of the product, D-arabino-3-hexulose-6-phosphate (HuMP), to give fructose-6-phosphate (FMP). The second phase involves the cleavage of the hexose phosphate skeleton to form glyceraldehyde phosphate (GAP) and dihydroxyacetone phosphate (DHAP) which can be looked at as the primary net product of the cycle. The last phase (the rearrangement phase) contains the reactions necessary for the regeneration of the acceptor molecule, RuMP.

Four variants of this cycle have been proposed. There are two rearrangement variants (Figures 1.5a, b), two cleavage variants, namely the Entner Doudoroff variant, which involves the enzyme 2-keto-3-deoxy-6-phosphogluconate aldolase (KDPG) and a variant in which fructose-1,6-bisphosphate aldolase is involved as the cleavage enzyme (FBP-variant) (Figure 1.6).

The enzymes needed for the cleavage in either variant have been demonstrated in *M. capsulatus* and *M. methanica* (Strom *et al.*, 1974). It

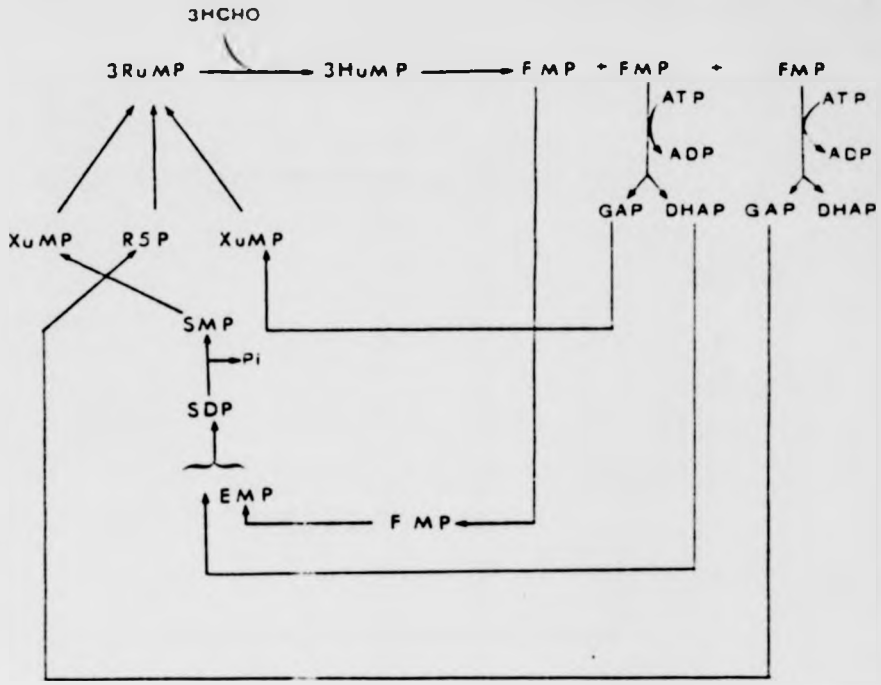


Figure 1.5a The RuMP cycle of formaldehyde fixation (sedoheptulose diphosphate (sda^+) variant)

(Modified from Quayle and Ferenci, 1978).

Abbreviations: HCHO: formaldehyde; HuMP: D-arabino-6-hexulose-phosphate; FMP: fructose-6-phosphate; GAP: glyceraldehyde phosphate; DHA: dihydroxyacetone phosphate; EMP: erythrose-4-phosphate; SDP: sedoheptulose diphosphate; SMP: sedoheptulose-7-phosphate; XuMP: xylulose-5-phosphate; Ru5P: ribulose-5-phosphate; RuMP: ribulose-5-phosphate.

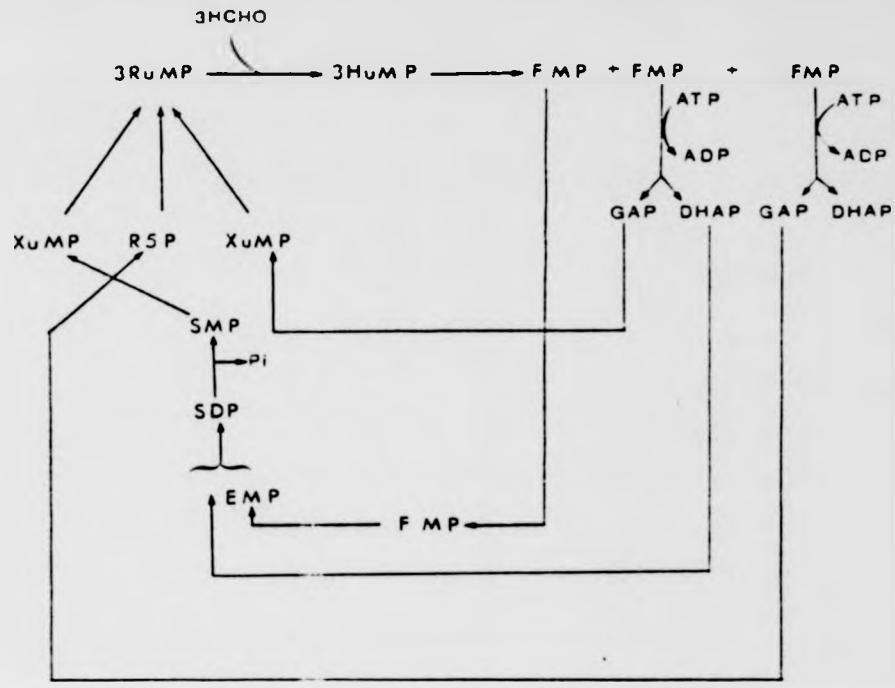


Figure 1.5a The RuMP cycle of formaldehyde fixation (sedoheptulose diphosphate (sda^+) variant)

(Modified from Quayle and Ferenci, 1978).

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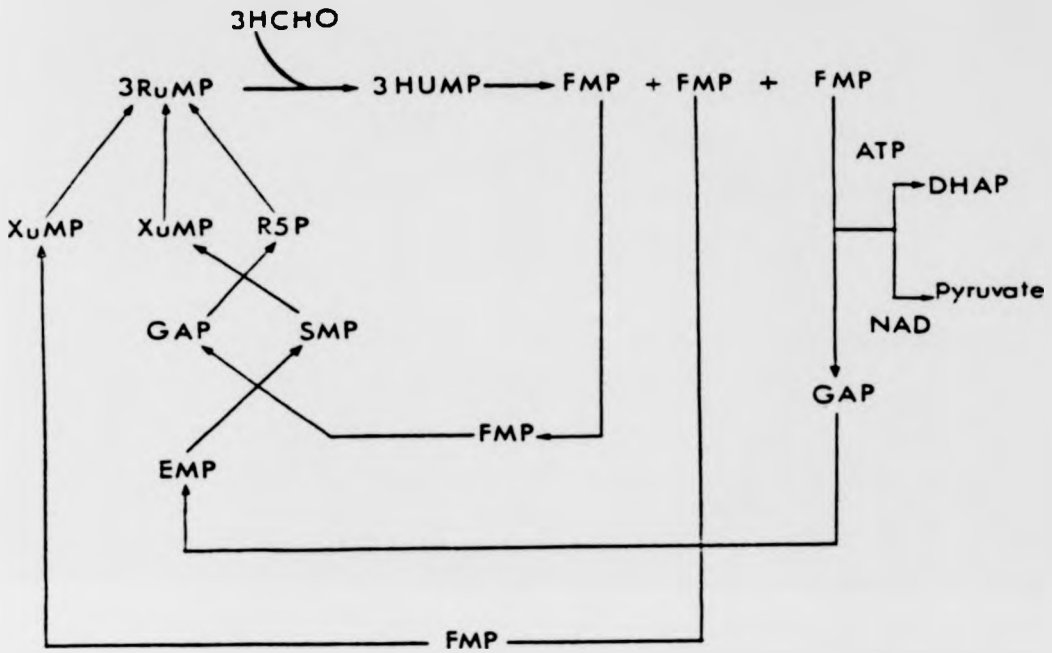


Figure 1.5b RuMP cycle of formaldehyde fixation (transaldolase tda$^+$, variant)

This variant involves transaldolase but not sedoheptulose diphosphate (Modified from Quayle and Ferenci, 1978).

Abbreviations are as for Figure 5a.

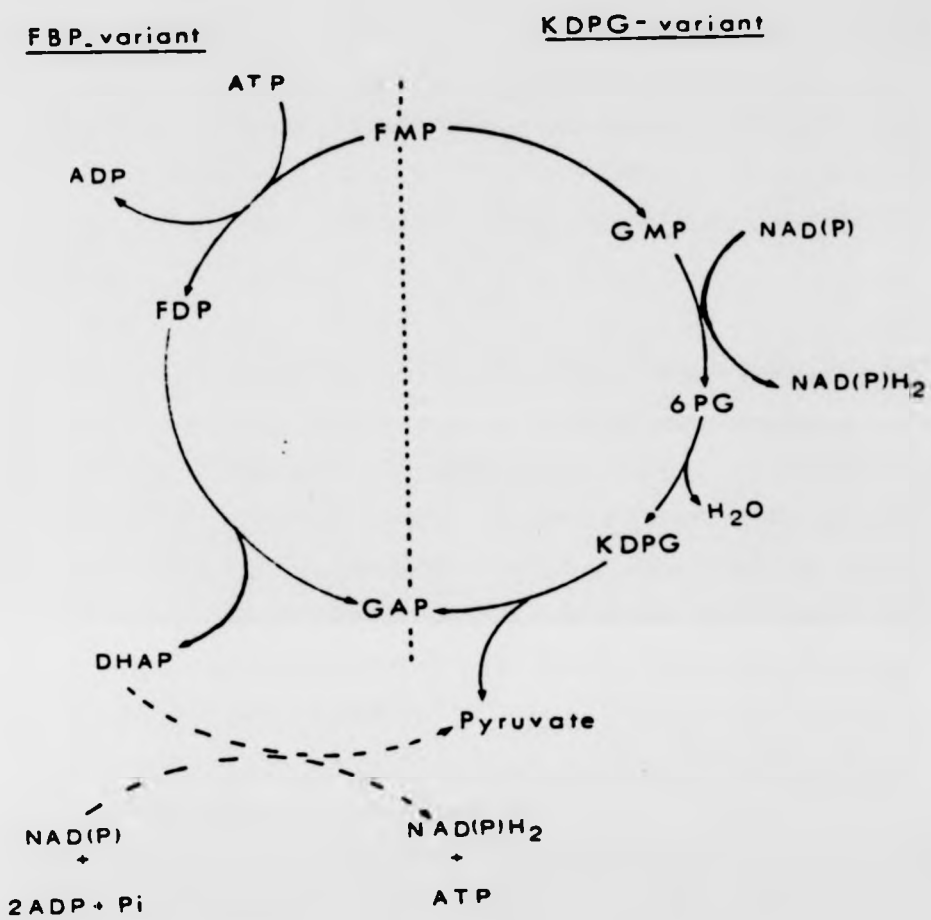


Figure 1.6 Alternative modes of cleavage of hexose phosphates

(After Quayle and Ferenci, 1978).

should be noted that in all the four variants reported, two reactions remain unique; the condensation of formaldehyde with Ribulose monophosphate (RuMP) catalysed by hexulose-phosphate synthase, and the isomerization of the product, D-arabino-3-hexulose-6-phosphate to yield fructose 6-phosphate, a reaction catalysed by hexulose phosphate isomerase

Recently a dihydroxyacetone pathway of formaldehyde fixation has been proposed for methanol grown yeasts as the eukaryotic counterpart of the bacterial-type RuMP cycle (van Dijken et al., 1978). In this pathway, a transketolase-type enzyme converts xylulose-5-phosphate and formaldehyde to GAP and dihydroxyacetone (DHA) which is phosphorylated by this kinase to DHAP. The regeneration of xylulose-5-phosphate occurs via the common rearrangement reactions. However, the detailed chemistry of this pathway is yet to be properly worked out.

1.3.1.1 Regulation of the RuMP pathway

Regulation of the RuMP pathway has been demonstrated in relatively few methylotrophs. Colby and Zatman (1975) showed that the specific activity of the key enzymes of the RuMP pathway were of the same order of magnitude in four facultative methylotrophs after growth on trimethylamine or glucose. They suggested that the enzymes are constitutive and are not sensitive to repression during growth of the organisms on heterotrophic substrates. However, there is as yet no report of this mode of regulation in obligate methylotrophs.

At the moment little is known about the regulation of the DHA cycle in yeasts. It may be of interest to note that the synthesis of the enzymes involved in methanol oxidation in the yeasts - Hansenula polymorpha and

Kloeckera sp. is regulated by a repression/derepression mechanism (Egli et al., 1980). The details of regulation of the RuMP pathway have as yet to be worked out.

1.3.2 The Serine Pathway of C_1 assimilation

The serine pathway of assimilation of reduced one-carbon compounds was originally proposed by Large and his co-workers in 1961 (Large et al., 1961). This pathway is known to operate largely during growth of many bacteria on C_1 compounds such as methane, methanol, methylated amines and formate. The essential reactions of the pathway are shown in Figure 1.7.

In this pathway, cellular carbon is derived, in part, from CO_2 by the action of phosphoenol pyruvate carboxylase (6) and the rest from the C_1 carrier methylene tetrahydrofolate (MTHF) by the action of transhydroxymethylase (1). The regeneration of glycine involves a cyclic series of reactions, and variations occur in the method of regeneration which is dependent on the presence or absence of isocitrate lyase (ICL). The final part of the pathway is the conversion of acetyl CoA to the C_3 and C_4 skeleton needed for the biosynthesis of cellular material. Two variants of the serine pathway have been suggested which differ in the way the C_2 acceptor molecule (glycine) is regenerated(Quayle, 1975). In organisms with the ICL^+ variant (Figure 1.8), the C_4 compound (malate) is activated to malonyl CoA and then cleaved to glyoxylate and acetyl CoA. Both glyoxylate and acetyl CoA are converted to glycine; acetyl CoA via the glyoxylate cycle in which the isocitrate lyase plays a key role. The ICL^+ variant has been demonstrated in a number of organisms including Pseudomonas MA (Hersh, 1975), and a number of obligate and facultative methane oxidizers (Colby et al., 1979).

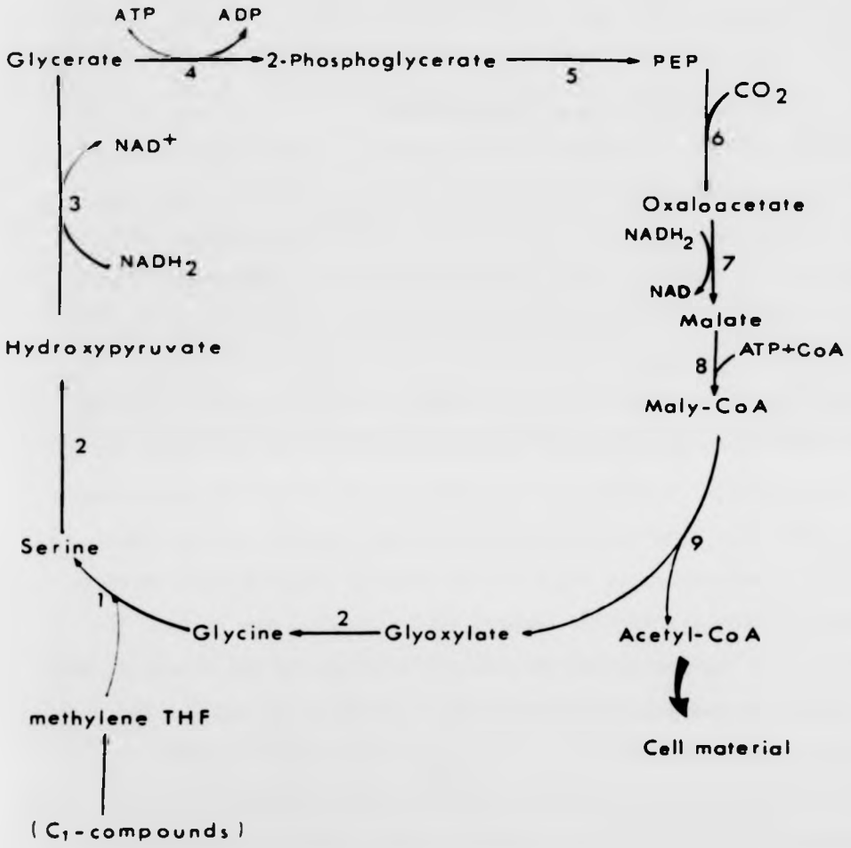


Figure 1.7 The serine pathway for methylotrophic growth on C_1 compounds

(After Anthony, 1975).

Enzymes:

1) serine transhydroxy methylase
2) serine glyoxylate aminotransferase
3) hydroxypyruvate reductase
4) glycerate kinase
5) enolase
6) phosphoenol pyruvate carboxylase
7) malate dehydrogenase
8) malate thiokinase
9) malyl CoA lyase

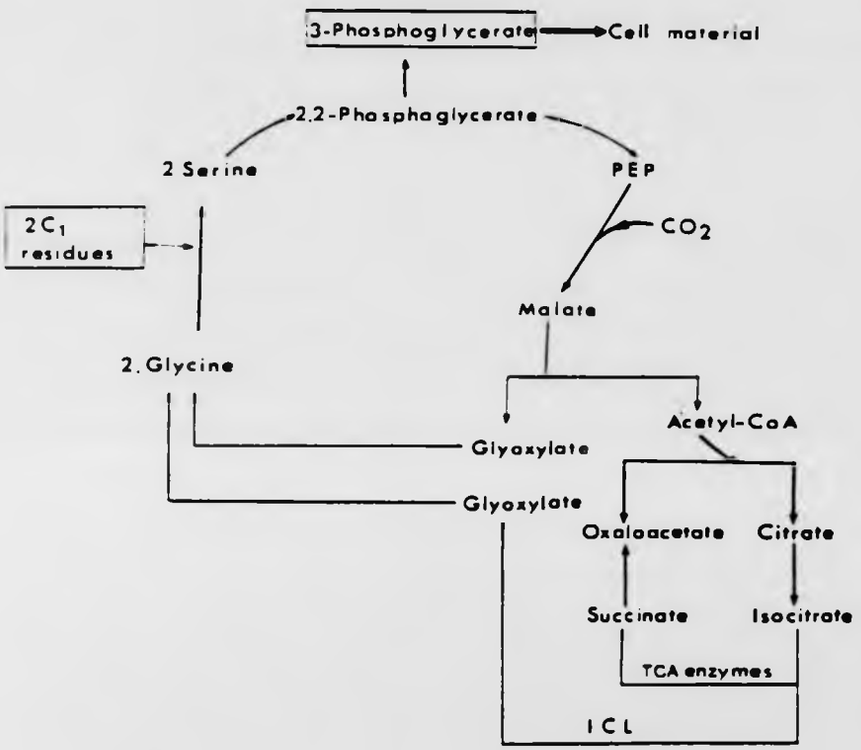


Figure 1.8 The serine pathway (ICL^+ - variant) of C_1 assimilation

ICL: isocitrate lyase

(Modified from Harder et al., 1973).

In the ICL$^-$ variant, the precise formulation of the regeneration of the C_2 acceptor molecule is not known. An example of an organism in which this variant of the serine pathway has been demonstrated is Pseudomonas AM1 (Anthony, 1975; Quayle, 1975). However, Anthony (1975) has suggested that Pseudomonas AM1 contains enzymes that oxidize acetyl CoA directly to glyoxylate (malate synthase may be involved) probably with glycollate as an intermediate.

It is of interest to note the detection of some of the serine pathway enzymes in several photosynthetic bacteria during heterotrophic growth of these organisms on particular compounds. For instance, Mue et al. (1964) demonstrated the presence of malyl CoA thiokinase and malyl CoA lyase in extracts of glutamate-malate grown cells of R. sphaeroides. However, the cleavage of malate to acetyl CoA (and presumably glyoxylate) by extracts of glutamate grown cells of R. rubrum needs reinvestigation to determine whether the ATP-, CoA and Mg^{2+} dependent cleavage (Stern, 1963) proceeds via malyl CoA. Although the serine pathway of one-carbon assimilation has not been fully established in photosynthetic autotrophs, the presence of the enzymes of this pathway in several of these organisms may suggest an evolutionary link between autotrophs and methylotrophs.

1.3.2.1 Regulation of the Serine Pathway

Regulation of the serine pathway is at present not well understood. Evidence thus far obtained with a number of organisms that use this pathway during growth on C_1 compounds indicate that the key enzymes of the pathway are co-ordinately induced, although the nature of the inducer is not known. Repression of the serine pathway enzymes in the presence of heterotrophic substrates has been reported (Dunstan et al., 1972; O'Connor and Hanson, 1977). Regulation at the molecular level remains to

be elucidated. However, transformation studies with mutants of Methylobacterium organophilum have shown physical linkage between many genes involved in the expression of several enzymes involved in C_1 metabolism by the serine pathway (O'Connor and Hanson, 1978).

1.4 The Energetics of C_1 Assimilation Pathways

The RuMP, ICL^+-serine and RuBP pathways of C_1 assimilation described, have different energy requirements. The permutation of two modes of cleavage and two modes of rearrangement leads to four possible variants of the RuMP cycle, and each of these variants has a different energy requirement (Table 1.2). To compare these with those of the ICL^+-serine pathway and RuBP cycle, all three cycles are normalized to pyruvate production using the established glycolytic steps (Figure 1.6) to convert triosephosphate and glyceric acid to pyruvate. The results of the normalizing process are shown in Table 1.3. Comparison of the RuMP pathway with those of the ICL^+-serine and the RuBP pathways shows the energetic cost to an organism of using CO_2 rather than formaldehyde as a C_1 unit in a net assimilation process, and this has considerable physiological implications. For instance, of the four possible variants of the RuMP pathway only the eda^+/sda^+ variant has not been found. This correlates with its relatively high energy requirement. There are two possible rearrangement sequences in the Calvin cycle; which of the two variants operate in autotrophically grown bacteria is largely unknown, although the tal^-/sda^+ is highly favoured.

It has been shown by a number of workers that the growth yield of organisms growing on C_1 compounds is affected by the pathway by which the

Table 1.2 Energy budgets for C_1 assimilation sequences

(After Quayle and Ferenci, 1978)

	Cleavage phase		Rearrangement phase				Energy Change		
Cycle	FDP-aldolase (fda)	KDPG aldolase (eda)	Trans aldolase (eda)	SDPase (sda)	Reactants	Products	$NAD(P)H_2$	FPH_2	ATP
RuMP	-	+	-	+	3HCHO	Pyruvate	+1	0	-3
	-	+	+	-	3HCHO	Pyruvate	+1	0	0
	+	-	-	+	3HCHO	Trios P	0	0	-2
	+	-	+	-	3HCHO	Trios P	0	0	-1
ICL + serine					2HCHO +1 CO_2	PGA	-2	+1	-3
RuBP			-	+	$3CO_2$	Trios P	-6	0	-9
			+	-	$3CO_2$	Trios P	-6	0	-9

Table 1.3 Energy budgets for C_1 assimilation sequences, normalized to pyruvate production

(After Quayle and Ferenci, 1978)

Cycle	Cleavage phase: FDP-aldolase (fda)	Cleavage phase: KDPG aldolase (eda)	Rearrangement phase: Trans aldolase (eda)	Rearrangement phase: SDPase (sda)	Reactants	Products	Energy Change: $NAD(P)H_2$	Energy Change: FPH_2	Energy Change: ATP
RuMP	-	+	-	+	3HCHO		+1	0	-3
	-	+	+	-	3HCHO	P	+1	0	0
	+	-	-	+	3HCHO	Y	+1	0	0
	+	-	+	-	3HCHO	R	+1	0	+1
						U			
ICL + serine					2HCHO	V	-2	+1	-2
					+1 CO_2	A			
						T			
RuBP			-	+	$3CO_2$	E	-5	0	-7
			+	-	$3CO_2$		-5	0	-7

compound is utilized. For instance, Goldberg et al. (1976) measured the growth yields of eight species of bacteria able to grow on C_1 compounds, and found that the molar growth yield values on methanol obtained for those bacteria using the RuMP pathway were higher than yields on those using the serine pathway. Similar observations have been reported by van Dijken and Harder (1975) and Anthony (1978). Furthermore, Anthony (1978) showed that the cellular yield of Paracoccus denitrificans growing on methanol by the RuBP pathway, was half the yield when methanol was assimilated via the RuMP pathway.

In conclusion, at this stage, the basic biochemistry of these assimilatory pathways is well understood, and emphasizes how the RuMP cycle touches upon virtually every pathway of bacterial carbohydrate metabolism. This property is believed (Quayle and Ferenci, 1978), to place the cycle in a unique position with respect to biochemical evolution.

1.5 Ribulose-1,5-Bisphosphate Carboxylase/Oxygenase

1.5.1 Historical Perspective

Ribulose-1,5-bisphosphate carboxylase/oxygenase (RuBisCO) was first discovered in 1947 by Wildman and Bonner (Wildman and Bonner, 1947), who observed a major protein in the soluble extracts of green leaves and called it Fraction I protein. Weissback and co-workers (Weissbach et al., 1956) purified the protein from spinach leaves and first demonstrated its enzymic nature by showing that it formed phosphoglyceric acid from carbon dioxide and ribulose bisphosphate. It was subsequently referred to as ribulose bisphosphate carboxylase (carboxymutase). This

protein often comprises more than 50% (w/w) of the total soluble leaf protein, which classifies it as the most abundant protein in the world (Ellis, 1979). The widespread occurrence of the ribulose bisphophate carboxylase/oxygenase soon became apparent when in 1959, Fuller and Gibbs demonstrated the presence of the enzyme in various photoautotrophic organisms. It was subsequently established that the enzymic entity of Fraction I protein was indeed RuBisCO and currently it is agreed that these two proteins are the same (Akazawa, 1979).

The carboxylation of ribulose bisphosphate (RuBP) by RuBisCO is a reaction of central importance in the Calvin cycle - the primary route by which inorganic CO_2 is assimilated into utilizable organic matter in autotrophic organisms (Stopani et al., 1955). Because of the key role of this enzyme in CO_2 fixation, photorespiration, and the diversity of organisms within which it is found, considerable interest has developed concerning its structure, function, regulation and molecular evolution.

1.5.2 Reactions catalysed by Ribulose bisphosphate carboxylase/ oxygenase

D-Ribulose-1,5-bisphosphate carboxylase [3-phospho-D-glycerate carboxylase (dimerizing), E.C.4.1.1.39] catalyses the condensation of one molecule of carbon dioxide, water and ribulose-1,5-bisphosphate (RuBP) to form two molecules of 3-phosphoglycerate (McFadden, 1973). The native protein functioning as a mono-oxygenase catalyses the oxygenolytic cleavage of RuBP to form a molecule each of phosphoglycollate and 3-phosphoglycerate (Figure 1.9). The carboxylase function of the enzyme has long been recognized, however the oxygenase activity was first proposed by Ogren and Bowes (1971) based on their observation that oxygen inhibited CO_2 fixation by RuBisCO. Andrews et al. (1973) and Lorimer

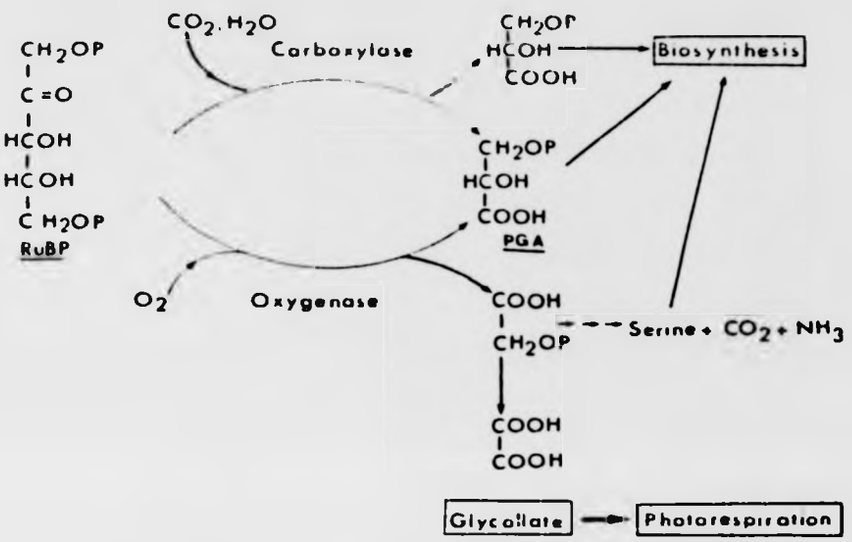


Figure 1.9 Metabolic significance of the reactions catalysed by ribulose-1,5-bisphosphate carboxylase/oxygenase

(Modified from Tabita, 1981).

et al. (1973) confirmed phosphoglycollate as product of the oxygenase reaction, and the fact that one mole of oxygen was consumed per mole of RuBP utilized. This oxygenase activity is thought to be responsible for photorespiration in higher plants (Andrews and Lorimer, 1978). Thus RuBisCO is an enzyme with dual function in catalysing two important steps in photosynthetic metabolism and is therefore most properly termed ribulose bisphosphate carboxylase/oxygenase. The oxygenase activity has been argued to be an inevitable consequence of the active site chemistry of the ribulose bisphosphate carboxylase (Andrews and Lorimer, 1978), which is supported by the observation that this enzyme has oxygenase activity even when isolated from anaerobic prokaryotes which do not evolve oxygen during photosynthesis.

The oxygenase activity of RuBisCO, which result in photorespiration, is regarded as the most important metabolic constraint on plant productivity (Hardy et al., 1978). In addition to the conventional biochemical approaches exploited to improve photosynthetic yield, recent applications of recombinant DNA technology promise to facilitate the genetic manipulation that may lead to improved plant productivity (Barber, 1984). The genetic aspect of RuBisCO and its possible application in agriculture will be considered later.

1.5.3 Molecular Properties of RuBisCO

1.5.3.1 Native Enzyme

RubisCO has been purified and characterized from a variety of sources and is most easily classified in terms of its quaternary structure (McFadden, 1973). Based on the holoenzyme molecular weight, the division of RuBisCO into three classes has been proposed by Anderson et al. (1968). These

classes are large (Mr 550,000), intermediate (Mr 240-360,000) and small (Mr 80-120,000). RuBisCO in all higher plants thus far examined (Kawashima and Wildman, 1970; Sigel et al., 1972; Akazawa, 1979), the green algae and the hydrogen bacteria (Kuehn and McFadden, 1969), some cyanobacteria (Akazawa, 1979) and photosynthetic halophiles (Tabita and McFadden, 1976) fall into the large molecular weight class. The enzymes from Thiobacillus denitrifican (McFadden and Denend, 1972), Chlorobium thiosulfatophilum (Tabita et al., 1974a), Pseudomonas oxalaticus (Lawlis et al., 1979), Rhodomicrobium vannielii (Taylor and Dow, 1980) and Methylococcus capsulatus (Bath) (Taylor et al., 1980) are of intermediate type. The only example of an enzyme with a small size is from Rhodospirillum rubrum having a Mr of about 120,000 (Tabita and McFadden, 1974b).

Although these three ranges have been considered adequate to describe the molecular size of the RuBisCO in nature (McFadden, 1973; McFadden and Tabita, 1974), there are some reported discrepancies. For instance, when the molecular weight of the RuBisCO from Synechococcus was determined by pore gradient electrophoresis, gel filtration and density gradient centrifugation, a value of Mr 430,000 was established (Andrews and Abel, 1981). However, further investigations employing equilibrium sedimentation yielded a value of Mr 530,000 (Andrews et al., 1981). This points to the fact that the assignment of molecular weight to the native enzyme, from which it is derived the quaternary structure, is markedly influenced by the determination method. Caution should be exercised therefore, when drawing conclusions from such experiments. Despite these differences in the molecular weights, enzymes from all classes have the same catalytic activity.

1.5.3.2 Subunit structure and composition

Ribulose bisphosphate carboxylase/oxygenase can be dissociated into subunits by treatment with sodium dodecyl sulphate (SDS) (Rutner and Lane, 1967), urea (Sugiyama and Akazawa, 1970) or p-mercuri-benzoate (Nirushima et al., 1973) and by mild acid treatment (Codd and Stewart, 1977). The enzyme dissociates, in most cases, into two types of subunits (as revealed by SDS polyacrylamide gel electrophoresis), the large (L) (Mr 55,000) and small (S) (Mr 15,000) which are normally present in a 1:1 ratio. In all eukaryotic photosynthetic organisms thus far examined, the holoenzyme has an octameric quaternary structure (Mr 550,000) being composed of eight pairs of large and small subunits (L_8S_8) (Kawashima and Wildman, 1970; McFadden, 1980). However, there is uncertainty regarding the spatial arrangement of the subunits.

The work of Baker and co-workers (Baker et al., 1975, 1977) using optical diffraction and electron microscopic techniques, suggest that the large subunits form a cube with the small subunits attached to the outside of this cube. Eisenberg et al. (1978) studied the purified enzyme from tobacco using electron microscopy and X-ray crystallography and proposed a bilayer model that indicated four large subunits stacked over another layer of four subunits. In this model, the actual location of the small subunits was not clear, though they predicted them to be arranged in the same plane as the large subunits situated peripherally in an array compatible with four-fold symmetry(see Figure 3, Miziorko and Lorimer, 1983). The model for RuBisCO (an L_8S_8 enzyme) from Alcaligenes eutrophus proposed by Bowien et al. (1980) differs from Eisenberg's model in that the large subunits are suggested to be U-shaped rather than globular . In Bowien's model, the small subunits are located above and below the layers of large subunits, rather than at the edge. However, the differences in the proposed models for the subunit arrangement of RuBisCO

are thought to be a consequence of conditions under which the crystals are generated (Tomimatsu and Donovan, 1981). They demonstrated a conformational change upon activation of the enzyme with bicarbonate and Mg^{2+}. The differences in the state of the enzymes used for studies by various authors may well have accounted for the differences in the models proposed. Detailed crystal analysis of activated and non-activated enzyme may thus provide some interesting findings that are critical for generating a catalytically active RuBisCO.

In prokaryotes, enzymes of different structures and consequently, molecular weights have been isolated (Table 1.4). Two main classes are usually recognised, described as the O- or T-type enzymes (Purohit and McFadden, 1977). The O-type enzymes consist of only the large subunits while the T-type enzymes have both the large and small subunits. The enzyme from Rhodospirillum rubrum is generally accepted to be an O-type, since it is a dimer of large subunits (Tabita and McFadden, 1974a, b). In the past, other bacterial enzymes have been reported with L_4, L_6 and L_8 structures (for review see McFadden, 1980). In the case of Rhodopseudomonas sphaeroides and R. capsulata two forms of RuBisCO with L_8S_8 and L_6 structures have been reported (Gibson and Tabita, 1977a, b). Whether the two enzymes from these organisms are completely different, or modified gene products, awaits further investigation, since Gibson and Tabita based their differences primarily on the kinetic properties of the two molecular forms of the enzyme.

The failure to detect the small subunits in RuBisCO from prokaryotic sources must be interpreted with caution. For instance, the Ribulose bisphosphate carboxylases from Anabaena cylindrica (Tabita et al., 1976) and Thiobacillus intermedius (Purohit et al.,(1976a)were reported to lack

Table 1.4 Comparisons of a number of RuBP carboxylase/oxygenases isolated from bacteria, green algae and higher plants

(Modified from McFadden, 1980)

Enzyme Source	Mr or S20W	Quaternary Structure	Oxygenase	Inhibition by 1 mM 6PGluA
Photosynthetic Bacteria				
Rhodospirillum rubrum				
Akazawa et al. (1970)	83,000	ND	ND	ND
Tabita & McFadden (1974b)	114,000	2L	yes	no
Chlorobium thiosulfatophilum				
Tabita et al. (1974a)	360,000	6L	ND	no
Rhodomicrobium vannielii				
Taylor & Dow (1980)	430,000	6L, 6S	yes	yes
Chromatium D				
Tabita & McFadden (1974c)	550,000	8L, 8S	yes	yes
Ecthiorhodospira halophilia				
Tabita & McFadden (1976)	600,000	8L, 8S	ND	yes
Thiocapsa reseopersicina		8L, 8S	yes	yes
Rhodopseudomonas sphaeroides				
Anderson et al. (1968)	14.5S	ND	ND	ND
Akazawa et al. (1970)	240,000	ND	ND	ND
Gibson & Tabita (1977a) I	550,000	8L, 8S	yes	yes
II	360,000	6L	yes	no
Chemosynthetic Bacteria				
Pseudomonas oxalaticus				
Lawlis et al. (1979)	360,000	6L, 6S	ND	yes
Thiobacillus intermedius				
Purohit et al. (1976a)	455,000	8L	ND	yes
Bowman & Chollett (1980)	550,000	8L, 8S	ND	yes
Thiobacillus A_2				
Charles & White (1976)	521,000	8L, 8S	ND	yes
Alcaligenes eutrophus				
Bowien et al. (1976)	18.2S 505,000	8L, 8S	yes	yes
Methylococcus capsulatus				
Taylor et al. (1981)	360,000	6L, 6S	yes	yes

Cyanobacteria				
Agmenellum quadruplicatum Tabita *et al*. (1974b)	456,000	8L	ND	ND
Anabaena cylindrica Tabita *et al*. (1976)	452,000	8L	ND	ND
Okabe & Codd (1980)		8L, 8S	ND	ND
Anabaena variablis Takabe *et al*. (1976)	18S	8L, 8S	ND	ND
Aphanocapsa sp Codd & Stewart (1977)	525,000	8L, 8S	yes	yes
Green Algae				
Euglena gracilis McFadden *et al*. (1975)	525,000	8L, 8S	yes	yes
Chlamydomonas reinhardi Givan & Criddle (1972)	530,000	8L, 8S	ND	ND
Chlorella fusca Lord & Brown (1975)	530,000	8L, 8S	yes	yes
Chlorella ellipsoidea	19S	8L, 8S	ND	ND
Halimeda cylindracea	18S	8L, 8S	yes	ND
Higher Plants				
Spinach Kawashima & Wildman (1970)	560,000	8L, 8S	yes	yes
Spinach beat Kawashima & Wildman (1970)	560,000	8L, 8S	ND	ND
Tobacco Kawashima & Wildman (1970)	525,000	8L, 8S	yes	ND
French bean Kawashima & Wildman (1970)	17.9S	8L, 8S	ND	ND

L = large subunit (50-58,000 daltons)
S = small subunit (11-18,000 daltons)
ND = not determined
6PGluA = 6 phosphogluconate

the small subunit. However, reinvestigation by Bowman and Chollet (1980) and Okabe and Codd (1980) demonstrated its presence. Codd and Stewart (1977) and Andrews and Abel (1981) have shown the relative ease by which the small subunit can be removed from the native enzyme by mild acid treatment, leaving a core of large subunits intact, which may be catalytically active. Hence the small subunits may be lost by the use of acid precipitation steps during purification procedures.

RuBisCO from many bacterial sources are of the T-type which consist of eight large and eight small subunits (McFadden, 1980). A number of bacterial RuBisCO have been shown to have an L_6S_6 structure (Lawlis *et al.*, 1979; Taylor and Dow, 1980; Taylor *et al.*, 1981; Andrews and Abel, 1981). The recent studies of Andrews *et al.* (1981) on the enzyme from *Synechococcus* sp. show that these reports must be viewed with caution. For example, Andrews and Abel (1981) proposed a hexameric L_6S_6 structure for the enzyme from *Synechococcus*, a structure based on its molecular weight as determined by pore-gradient and gel filtration. When the molecular weight of the enzyme was reinvestigated using equilibrium sedimentation, a value of Mr 530,000 was obtained, which is within the range for an L_8S_8 structure. Moreover, electron microscopic studies revealed a four-fold symmetry which is characteristic of an L_8S_8 structure. It should be noted that the structure of the RuBisCO enzyme from *Rhodomicrobium vannielii* has been reinvestigated using the equilibrium analytical centrifugation method, and the results obtained are consistent with the hexameric L_6S_6 structure (Dow, unpublished observations).

It is evident from these surveys that considerable doubts surround the nature of the enzyme from some prokaryotic organisms. In the face of

these conflicting results, therefore, the application of a rapid, one-step purification method as developed in the Department of Biological Sciences, University of Warwick, for the isolation of RuBisCO from a spectrum of prokaryotes, would be useful in solving these inconsistences.

The primary structures of the large and small subunits of RuBisCO in terms of the amino acid composition and sequence has been an area of considerable research interest in recent years. This is necessary not only from the point of view of establishing evolutionary relationships between photosynthetic prokaryotes and eukaryotes, but is also of use in the genetic manipulation of RuBisCO in relation to its carboxylase-oxygenase activity, i.e. with a view to increasing plant productivity.

Comparison of the amino acid composition of large subunits of eukaryotic RuBisCO show that they are very similar (Akazawa et al., 1978). Recently the genes coding for the large subunit in maize (McIntosh et al., 1980), spinach (Zurawki et al., 1981), Chlamydomonas (Dron et al., 1982) have been cloned and sequenced allowing for the amino acid sequence to be deduced. All of the evidence thus far obtained shows a high degree of homology (>85%) - a total of 475 common residues in the large subunits of eukaryotic photosynthetic organisms. A high degree of homology has also been observed between the plant large subunit and the cloned sequence of the Synechococcus enzyme (Reichelt and Delaney, 1983). Of special interest is the complete sequence conservation at the regions which have been implicated in catalysis and activation.

In contrast to the homology that characterizes the primary structure of the large subunits of the plant enzymes, it has been observed that the Rhodospirillum rubrum enzyme differs significantly in amino acid

composition (Akazawa et al., 1978). This suggests that the prokaryotic and eukaryotic enzymes may not be very closely related in the evolutionary sense. However, Hartman et al. (1982) and Nargang et al. (1984) have sequenced more than 80% of the primary structure of the R. rubrum enzyme and showed only 28% of the residues to be identical to those reported for the plant enzyme. Although only a small degree of homology exists between the large subunits of the plant and the R. rubrum enzyme, two regions stand out as being highly conserved. Lysines residues at positions 175 and 334 in the spinach enzyme were identified as being active site residues on the basis of affinity labelling studies with 3-bromo-1,5,dihydroxy-2-butanon-1,4-bisphosphate (Stringer and Hartman, 1978), and with N-bromoacetylethanolamine phosphate (Schloss et al., 1978). A similar situation was established for the R. rubrum enzyme, hence the speculation that the catalytic site is conserved. Nargang et al. (1984) have shown that the two amino acid residues (Lys-166 and lys 329), which were positioned in or near the active site by affinity labelling were also found in the highly conserved sequence. Similarly lys-191 was found to be strongly homologous to the region around lys-201 of the spinach enzyme. This residue has been shown to react with CO_2 to form a carbamate which results in the activation of the spinach enzyme (Lorimer, 1981a). Since the R. rubrum enzyme also undergoes CO_2 activation, it seems likely that the apparent sequence homology between the spinach large subunit and the R. rubrum enzyme, reflects an identical chemical basis for the functional equivalence. All evidence thus far shows that, although the homology between the R. rubrum enzyme and the plant enzyme is low, the active and catalytic sites are highly conserved. Thus the suspicion (Lorimer, 1981b) that the R. rubrum enzyme may have evolved independently may not be valid.

In contrast to the large subunit, the small subunits of RuBisCO from different species have different amino acid composition (Takabe and Akazawa, 1975; Akazawa et al., 1978). The primary structures of the small subunits from Pisum sativum (Takuri et al., 1981), spinach (Martin, 1979) and Tobacco (Muller et al., 1983) have been determined by protein sequencing techniques, while those from soyabean and pea have been determined by cloning and sequencing (Bedbrook et al., 1980; Berry-Lowe et al., 1982). Available data shows that there is about 70% homology between the sequence of mature spinach, soyabeans and pea small subunits. Apparently during phylogenetic evolution of the RuBisCO molecule, the genetic information has been conserved in the chloroplast for the large subunit, while genetic variation occurred with the nuclear-coded small subunit.

Currently, there is no information about the amino acid sequences of the small subunits from prokaryotic RuBisCOs. However, cloning and sequencing of the small subunits from various sources, with the view of finding the highly conserved region, may provide informations pertinent to the functioning of the small subunits.

The case for subunit heterogeneity:

When examined by iso-electric focussing, the subunits of RuBisCO were found to be heterogeneous (Kung et al., 1974). Heterogeneity has also been observed for the large subunit of the Alcaligenes sutrophus enzyme (Purohit and McFadden, 1976, 1977). However, Bedbrook and Kolodner (1979) indicated that charge heterogeneity must be interpreted with caution. Indeed, chemical analysis of the iso-electric variants of the large subunit from Nicotiana sp. failed to detect any differences between

them. Gray et al. (1978) concluded that the variants resulted from the modification of a single gene product. Recently, O'Connel and Brady (1982) indicated that the charge heterogeneity arises artifactually during the alkylation procedure that preceeds iso-electric focussing. Thus far the weight of evidence points to the large subunit being homogeneous, i.e. the product of a single gene (Ellis, 1981).

In most eukaryotes, the small subunit is encoded by a small gene family in the nucleus (Broglie et al., 1983). Iso-electric focussing has revealed the presence of charge variants in the mature small subunit from pea (Takuri et al.. 1981), but no heterogeneity has been observed in the amino acid sequence. However, proof of small subunit heterogeneity has been obtained for the spinach and tobacco small subunits by amino acid sequencing and tryptic peptide finger print analysis (Martin, 1979; Gray et al., 1978). The occurrence of heterogeneity is worrying in terms of the kinetic properties of the enzyme, i.e. kinetic constants determined for heterogeneous enzyme preparation represent average values for the whole population of molecules.

Immunological techniques have been used to probe for homology between enzymes from different sources. Immunological comparisons of RuBisCO from divergent sources have been summarized by McFadden (1973). Lord et al. (1975) have reported that antiserum to the enzyme from Euglena gracilis inhibited RuBisCO from Chlorella fusca and four species of cyanobacteria, in addition to forming precipitates with enzymes in extracts from all these sources. Generally, immunological studies indicate the homologous nature of the large subunits from different sources (McFadden, 1973). However, it is of interest to note that the antibodies to the R. rubrum enzyme failed to yield precipitates with

RuBisCO from any source tested, including spinach, green algae, cyanobacteria, the green and sulphur bacteria; nor do they inhibit the enzymes from Hydrogenomonas eutropha (Tabita and McFadden, 1974b). Similarly, Gibson and Tabita (1977c) showed that antiserum directed against Form II (L_6) RuBisCO from Rhodopseudomonas sphaeroides showed no cross-reactivity towards the Form I enzyme from the same organism. In addition, antibody against the Form II enzyme failed to inhibit the Form I enzymic activity. However, failure to demonstrate cross-reactivity by antiserum directed against one protein towards another may be a function of the small subunit masking the antigenic determinants on the large subunit.

1.5.3.3 Subunit Function

Evidence has been presented to show that the catalytic site is associated with the large subunit (McFadden and Tabita, 1974a, b). Perhaps the most convincing argument for the catalytic role of the large subunit was provided by the isolation of homogeneous RuBisCO (a dimer of large subunits) from the purple non-sulphur bacteria, Rhodospirillum rubrum capable of fixing CO_2 (Tabita and McFadden, 1974a). The enzyme from R. rubrum has been shown to undergo activation by CO_2 and Mg^{2+} via carbamate formation (Whitman et al., 1979; O'Leary et al., 1979), and it catalyzes both the carboxylation and the oxygenation reaction (McFadden, 1974). These results suggest that all of the amino acids involved in these processes are present on the large subunit. Two further lines of evidence support the assignment of this role to the large subunit of the L_8S_8 enzyme:

i) Lorimer and Miziorko (1980a)and Lorimer (1981b) have trapped the activator, CO_2, on the large subunit of RuBisCO from plants, and have

also showed that the ε-amino group at lysine 201 linked the activator CO_2 to the protein.

ii) Miziorko et al. (1982) have probed the activator cation binding site using Cobalt; the metal ion was shown to bind directly to amino acids of the large subunits of the spinach enzyme.

The function of the small subunit, however, has not been established. Some authors have suggested that the small subunits play a hitherto unrecognized regulatory role (Takabe and Akazawa, 1973). It has also been suggested that the small subunits house the activation and effector sites of the enzyme (Jensen and Bahr, 1797), while others believe that the small subunit may be a positive factor needed for the synthesis of the large catalytic subunits (Ellis, 1975). It has also been suggested that the binding of the oligomeric large subunit core in a 1:1 stoichiometry with the small subunits results in a more active conformation (Andrews and Abel, 1981; Andrews et al., 1981). However, substantive evidence for these views is lacking and the function of the small subunit is at the moment a mystery. Almost certainly these questions will have to be approached from a genetical standpoint.

1.5.4 Catalytic Properties of RuBisCO

For many years following the discovery of RuBisCO, its kinetic properties were widely considered to be anomalous. Specifically the apparent Michaelis constant for carbon dioxide (Km [CO_2]) *in vitro* was considerably higher than the equilibrium concentration of CO_2 in the aqueous environment of autotrophic species to account for the *in vitro* role of this enzyme. For example, Hatch and Slack (1970) observed that the concentration of CO_2 in a solution in equilibrium with air and at a temperature between 20° and 30°C will be about 8 μM, an order of magnitude lower than the Km (CO_2) of 450 μM for spinach RuBisCO reported in 1969 by Cooper and co-workers. Recent investigations have however, shown that the purified RuBisCO which showed high Km values for CO_2 contained kinetically inactive form. Bahr and Jensen (1974a) demonstrated that upon rapid breakage of spinach chloroplasts with a hypotonic medium, a form of RuBisCO having a low Km for CO_2 (11-18 μM) can be isolated, and it rapidly converts to a high Km (CO_2) form (20-25 mM HCO_3) similar to the purified enzyme.

The reports of a form of RuBisCO with an apparent low Km for CO_2 which would account for the observed *in vivo* rate of CO_2 fixation in plants (Bahr and Jensen, 1974a, b; Badger and Andrews, 1974) were shown to be due to an induced activation by appropriate physical conditions and the concentrations of CO_2 and Mg^{2+} (Lorimer *et al.*, 1977). Furthermore, the order of addition of reagents to the enzyme affects the time course and the subsequent reaction. For maximum activity, the enzyme must be incubated with CO_2 and Mg^{2+} prior to initation of the reaction with RuBP (Kuehn and McFadden, 1969; Tabita and McFadden, 1974a; Lorimer *et al.*, 1976). Lorimer *et al.* (1976) have also suggested that the order of

addition of CO_2 and Mg^{2+} and their concentration are important for the activation of this enzyme.

1.5.4.1 Activation of RubisCO

The essential details of the molecular mechanism for the activation of RuBisCO by CO_2 and divalent metal ions (Me^{2+}) has been worked out and is represented in the Figure 1.10 (Miziorko and Lorimer, 1983).

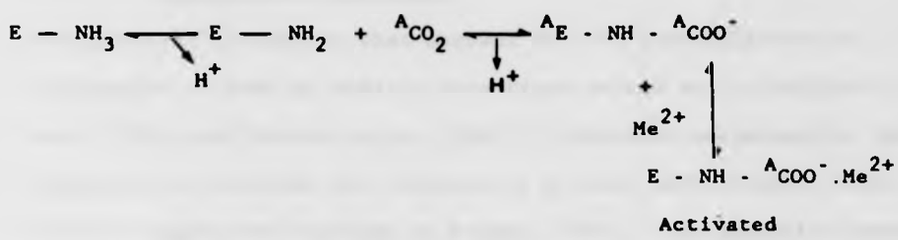


Figure 1.10 Mechanism of activation of RuBisCO by CO_2 and Me^{2+}

In this scheme, activation involves carbamate formation with the epsilon amino group of lysine 201 on the large subunit (Lorimer and Miziorko, 1980a; Lorimer, 1981a) followed by the binding of a metal ion to form an active enzyme complex $E.CO_2.Mg^{2+}$. It is this complex that catalyses the carboxylation reactions. The activity of this complex is dependent upon the concentration of CO_2, Mg^{2+} and pH of incubation (Lorimer et al., 1976).

Kinetic and spectroscopic studies (Lorimer et al., 1976; Miziorko, 1979) have established that the addition of CO_2 preceded the addition of Me^{2+}. Although there is evidence to suggest that bicarbonate (HCO_3) is the species of CO_2 taken up by whole cells of R. rubrum (Christeller and Laing, 1978), Cooper et al. (1969) have shown that CO_2 is the active

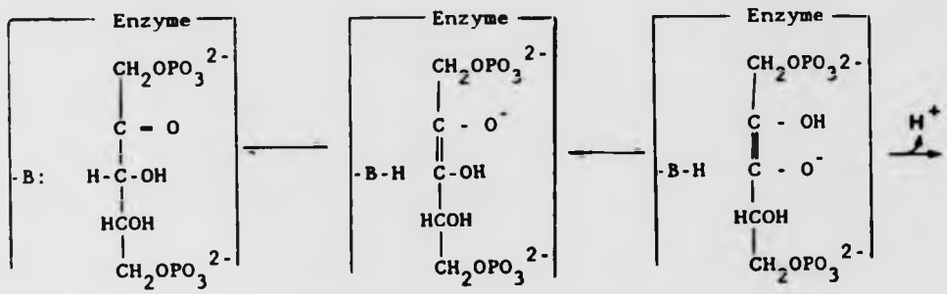
species of RuBisCO reaction. Moreover, competitive binding and kinetic turnover experiments of Miziorko (1979) and Lorimer (1979) have established that the CO_2 molecule which participates in activation is not the same as that which becomes fixed. Activation by CO_2 and Me^{2+} is a property that appears to be common to all RuBisCOs regardless of their taxonomic origin.

1.5.4.2 Mechanism of Catalysis

The essential mechanisms that account for the carboxylation and oxygenation of RuBP by RuBisCO were first worked out by Mullhofer and Rose (1965) and Fielder et al., 1967. A detailed mechanism for the carboxylation reaction was proposed by Lorimer and Miziorko (1980a, b) and the oxygenation reaction by Kosman (1978). The essential features of these proposed mechanisms are shown in Figures 1.11a and 1.11b.

The key elements of this scheme, namely, the tautomeric form of enediol form of RuBP intermediates were suggested by Calvin (1954) and still appear substantially correct. The reaction begins with the removal of a proton from C-3 of RuBP to form the enediol (step 1). The nature of the base B which brings this about is not quite known, although Hartman et al. (1978) suggested lysl residues, 176 and 334, as being within the active site domain. The enediol is then attacked by CO_2 (carboxylase reaction) and the resulting 2-carboxy-3 keto species is rapidly attacked at the C-3 position by a water molecule, yielding a D-phosphoglycerate molecule from the bottom half of the intermediate and the aci-acid form of phosphoglycerate from the top half. Addition of a proton to the aci-acid yields a second molecule of the product. The experimental evidence that supports this sequence of events is substantial and has been detailed in the Review by Miziorko and Lorimer

Figure 1.11a Proposed Mechanism for the carboxylation of RuBP (after Lorimer and Miziorko, 1980a)



tautomeric forms of enediol intermediates

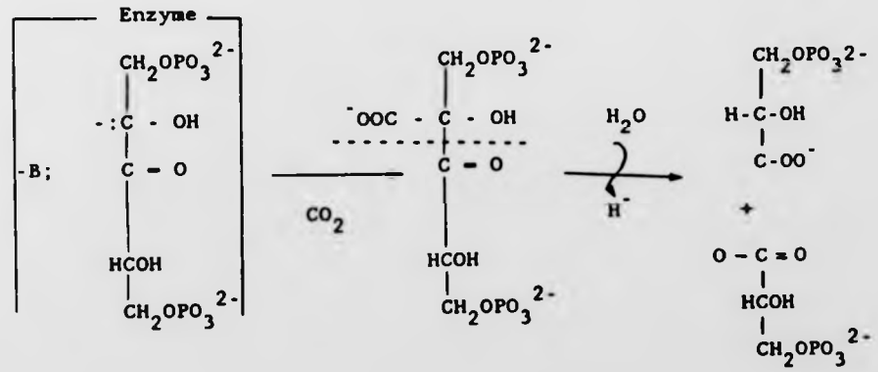
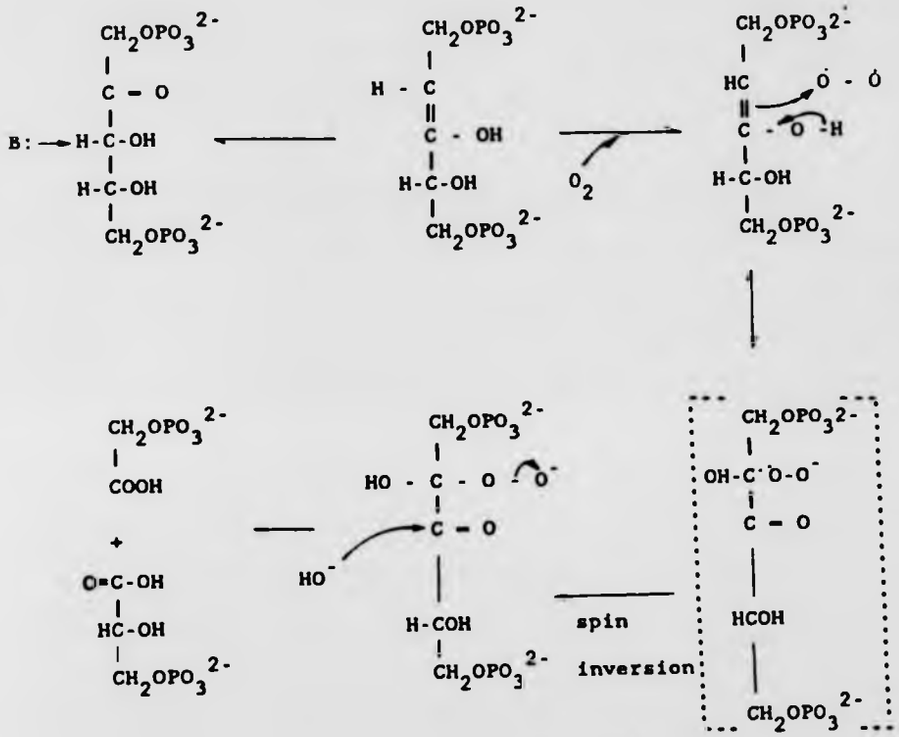


Figure 1.11b Mechanism for the oxygenation of RuBP (after Kosman, 1978)



(1983).

A mechanism analogous to that proposed for carboxylation has been offered to explain the oxygenation of RuBP (Figure 1.11b). However, it has been established that with $^{18}O_2$, one atom of ^{18}O is incorporated into the carboxylate group of phosphoglycollate; the other ^{18}O is apparently lost into the medium (Lorimer et al., 1973).

1.5.4.3 Natural variation in Catalytic Activity of RuBisCO

One aspect of the catalytic properties of RuBisCO which is encouraging at the moment, is the variation in the kinetic properties of this enzyme isolated from different organisms. The only aspect of RuBisCO that has so far proved to be invariant is that carboxylase activity is always associated with oxygenase activity. Other aspects show variation between species, especially with respect to the Km (CO_2) and to the substrate specificity factor (Laing et al., 1974). The substrate specificity factor defines the ratio of carboxylation to oxygenation catalysed by RuBisCO. This can be represented by the equation:

$$v_c/v_o = (VcKo/VoKc)([CO_2]/[O_2])$$

where v_c and v_o are the velocities of carboxylation and oxygenation respectively, Vc and Vo the maximal velocities of the two reactions, Kc and Ko the Km for CO_2 and oxygen. From this equation, it can be seen that at any given concentration of CO_2 and O_2, the relative rates of carboxylase and oxygenase reaction will be determined by the specificity factor (VcKo/VoKc). The greater its value the greater is the specificity for CO_2. The kinetic parameters of RuBisCO have been determined for a variety of organisms by Jordan and Ogren (1981, 1983). They showed that

the Km (CO_2) decreases by at least one order of magnitude from the photosynthetic bacteria to higher plants; this was less marked for the substrate specificity factor. Jordan and Ogren interpreted the changes in terms of the evolution of RuBisCO to compensate for the geological shift from an atmosphere containing high CO_2 and low O_2 concentrations to one consisting of low CO_2 and high O_2 concentrations.

It is clear from this work that during the course of evolution, modification of RuBisCO has occurred, improving the efficiency of carboxylation. How far such changes can be artificially continued to improve plant productivity is the key question to be answered in the future.

1.5.5 Synthesis of RuBisCO

In recent years, studies have been carried out concerning the mechanism of the biosynthesis of ribulose bisphosphate carboxylase from various plant origins, for example pea (Smith and Ellis, 1979), rye (Feierabend and Wildner, 1978), soyabean (Barraclough and Ellis, 1979) or green algae such as Chlamydomonas reihardtii (Schmidt et al., 1979). All evidence thus far obtained shows that the large subunits are the major products of the chloroplast genetic system; the messenger RNA being translated on free chloroplast ribosomes (Ellis, 1981). The small subunit is encoded for by a small gene family in the nucleus (Broglie et al., 1983). It is believed that the small subunit is first synthesized as a precursor (P20) in the cytoplasm, and then transported by an ATP-dependent post-translational mechanism across the chloroplast envelope into the chloroplast where it is processed to become a "matured" polypeptide. The

final interaction between the large and small subunits to form catalytically active enyzme occurs in the chloroplast stroma (Smith and Ellis, 1979). The current model for the synthesis of RuBisCO in the plant system is shown in Figure 1.12.

There are different views with regard to the interaction of the two subunits molecules during their formation. In contrast to several studies showing tightly coupled synthesis of the two subunits (Ellis, 1975; Nirushima and Akazawa, 1978), there is an indication that under certain conditions such as those occurring in rye plants (Feierband and Wildman, 1978) or isolated soyabean leaf cells (Barraclough and Ellis, 1979), small subunit is synthesized independently of large subunit.

On the other hand, there is only very scanty information available concerning the biosynthesis of the RuBisCO molecule in photosynthetic prokaryotes in which intracellular compartments such as the chloroplast and nucleus do not exist. However, it has been suggested that in photosynthetic bacterium, Chromatium vinosum, the biosynthesis of the large and small subunits is completely synchronized and may be regulated by identical mechanisms (Kobayashi and Akazawa, 1982). Certainly, the mechanism of biosynthesis of RuBisCO in prokaryotic organisms will be better understood by employing immunological techniques coupled with pulse-chase labelling experiments.

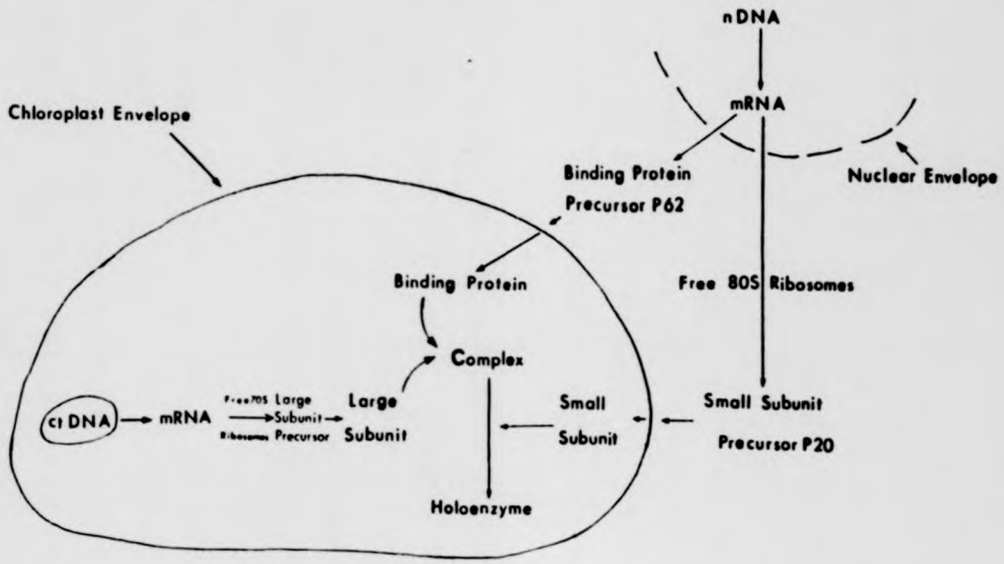


Figure 1.12 The Synthesis Of RuBisCO in the Plant system (After Ellis & Gatenby, 1984)

1.5.6 Regulation of RuBisCO

1.5.6.1 Regulation of Enzyme Activity

Although the molecular mechanism of activation and catalysis of RuBisCO is largely understood, the physiological role it plays in regulating enzyme activity *in vivo* is less clear. However, a number of compounds and factors are known to influence the enzyme activity *in vitro*.

RuBisCO activity is highly dependent upon added Mg^{2+} (Kawashima and Wildman, 1970). Other divalent cations, notably Mn^{2+}, Ni^{2+} and Co^{2+} exhibit the capacity to partially substitute for Mg^{2+} in the reaction catalysed by T-type RuBisCOs. The O-type enzymes from *R. rubrum* and Form II from *R. sphaeroides* are specific for Mg^{2+} (Tabita, 1981). As already pointed out, Me^{2+} functions in the activation reaction although the role it plays has not been properly established. It has been suggested that the Me^{2+} co-ordinates to, and stabilizes the carbamate (Lorimer, 1981a; Lorimer and Miziorko, 1981). This view is supported by the acidic nature of the amino acids adjacent to lysine 201 (Miziorko and Lorimer, 1983). Since carbamate is formed in this region, the presence of the acid groups is likely to make the formation (of a carbamate at lysine 201) thermodynamically unfavourable, since this introduces negative charge into an already anionic region. Hence the co-ordination of Me^{2+} to the carbamate and possibly to some of the adjacent groups will neutralize the negative charges and thus stabilize the active enzyme complex.

It has been reported that when RuBisCO is presented with physiological levels of one of its substrates, RuBP, in a medium in which either carbon dioxide and HCO_3^- or Mg^{2+} ions have been kept at a very low concentration, the enzyme is inactivated (Chu and Bassham, 1973, 1975). Jordan and

Chollet (1983) showed that RuBP is a potent and weak inhibitor of the rate of CO_2/Mg^{2+} activation of RuBisCO purified from spinach leaves and Rhodospirillum rubrum respectively. The reasons for the differences in sensitivity to inhibition by substrate, RuBP, were associated with the different binding constants (K_D RuBP) and the dissociation of RuBP from the native enzymes. Jordan and Chollet concluded that strong inhibition of CO_2/Mg^{2+} activation in the spinach enzyme is mediated by the tight binding and slow release of RuBP, which prevents activator CO_2 and Mg^{2+} from binding to the protein. Weak inhibition of activation in the R. rubrum enzyme results from a larger K_D value and the rapid release of RuBP, which allows the activators, CO_2 and Mg^{2+}, to bind to the free enzyme between substrate binding events. However, it has been shown that the inhibitory effect of RuBP is not observed when the enzyme from plant or bacterial systems is preincubated with high CO_2 and Mg^{2+} concentration before the initiation of the reaction by addition of RuBP (Chu and Bassham, 1975).

In addition to substrate inhibition/activation of RuBisCO activity, a number of metabolites (effectors) such as 6-phosphogluconate, NAD(P)H and fructose-1,6-diphosphate (FBP) have been demonstrated to have effects on the activity of RuBisCO isolated from spinach leaves (Chu and Bassham, 1972, 1975; Tabita and McFadden, 1972). In general it is found that only the T-type enzyme are inhibited by 6-phosphogluconate while the O-type enzymes from R. rubrum, C. thiosulfatophilum , T. denitrificans and the Form II RuBisCO from R. sphaeroides are not affected (Tabita and McFadden, 1794a; Tabita et al., 1974a; McFadden and Denend, 1972; Gibson and Tabita, 1977a). However, Chu and Bassham (1975) showed that the T-type enzyme purified from spinach was activated by 0.01M 6-phosphogluconate and 0.5 mM NADPH when added prior to the addition of

RuBP during pre-incubation. This they interpreted as 6-phosphogluconate preventing the inhibitory binding of RuBP to allosteric sites on the enzyme. On the other hand when the enzyme, following incubation with HCO_3 and Mg^{2+}, is presented with RuBP and 6-phosphogluconate, competitive inhibition was observed with respect to RuBP (Chu and Bassham, 1975). Based on their studies on the order of addition of substrates and effectors, concentration effects and kinetics, Chu and Bassham proposed allosteric regulation, combined with competitive inhibition. This then suggests a complex regulatory mechanism for RuBisCO. The non-sensitivity of the O-type enzymes to 6-phosphogluconate, may correlate with the absence of small subunits and also suggests a different catalytic site topography to that of the large molecular (T-type) RuBisCOs.

However, care should be taken before attributing any physiological significance to the influence of these effectors. For instance, Lorimer et al., (1978) pointed out that in order for any putative effector to be physiologically relevant, it needs to be present in vivo in the amounts that approach or exceed the in vivo concentration of RuBisCO active sites. The concentration of RuBisCO active sites within the chloroplast is on the order of 4 mM, but the pools of most of the metabolites reported to influence the activity of RuBisCO enzyme in vitro are simply not large enough to satisfy this criterion (Lorimer et al., 1978). Secondly, the binding of effectors at one or more allosteric sites, dinstinct from catalytic sites, e.g. (Chu and Bassham, 1975) is frequently invoked to account for the observed phenomenon. However, Badger and Lorimer (1981) and McCurry et al. (1981) have demonstrated that the various effectors interact with the enzyme at a single site, the catalytic site for RuBP. It follows therefore, that an enzyme molecule cannot be simultaneously catalytically competent and activated by one of

the effectors, since the latter involves occupancy of the substrate binding site. This does not prevent the existence of a metabolite that promotes the activation reaction by interaction at a site distant from the catalytic site. The physiological significance of the effects of sugar phosphates on some RuBisCO remain in doubt. The influence of various sugar phosphates on the activity of RuBisCO from T. novellus (McCarthy and Charles, 1973) may require further investigation in view of the fact that these phosphates have no effect on the enzyme from Pseudomonas facilis (McFadden and Tabita, 1974).

It has been suggested that the activity of RuBisCO can be modulated by light. For example, evidence has been presented for an indirect light effect, mediated via increased alkalination of the chloroplast stroma and increased stromal Mg^{2+} concentrations (Jensen et al., 1978). Direct effects of light on the activation of RuBisCO enzyme from photosynthetic eukaryotic organisms have been reported (Daley et al., 1978). In contrast to the situation in higher plants, visible light has been implicated in the photo-inactivation of RuBisCO cyanobacterial cells (Stewart and Codd, 1980 and in the extracts from purple sulphur bacteria (Asami and Akazawa, 1978), cyanobacteria (Codd and Stewart, 1980). The physiological significance of light in the regulation of RuBisCO and on Calvin Cycle has been considered (see Section 1.2.14.3).

In view of the differences in the kinetic properties of T- and O-type ribulose bisphosphate carboxylase/oxygenase, a number of authors have implicated the small subunit in the regulation of activation and catalysis of this enzyme. For instance, Tabita (1981) indicated that the binding of the small subunit to the large subunits may provide a conformation that results in the capacity for metal ions in addition to

Mg^{2+} and Mn^{2+} to support activation or catalysis. The binding of the small subunits to large subunits is also being suggested to result in a more active enzyme conformation (Andrews and Abel, 1981). However, evidence for the involvement of small subunits in the regulation of RuBisCO are not substantive and comparative regulatory properties of enzymes with and without small subunits may be worthwhile.

1.5.6.2 Control of RuBisCO Synthesis

Despite the obvious significance of ribulose-bisphosphate carboxylase/-oxygenase in CO_2 assimilatory pathways and in agriculture, little is known about its regulation at the molecular level. For instance, in prokaryotic systems it is not known how many genes are involved or whether they comprise an operon. The current views concerning the regulation of the synthesis of RuBisCO in plant systems have been reviewed by Ellis and Gatenby (1984). Studies on the regulation of RuBisCO synthesis in prokaryotes have always been approached from a physiological point of view.

In general, the synthesis of RuBisCO in facultative autotrophs is efficiently regulated. A detailed study of the regulation of the synthesis of RuBisCO in Pseudomonas oxalaticus OX1 (Dijkhuizen and Harder, 1979a) has shown that RuBisCO in this organism is very sensitive to repression in the presence of heterotrophic substrates. This repression has been studied in detail and the patern of results indicate the presence of a repression regulatory system, i.e. the concentration of an unknown intracellular repressor molecule(s) controls the amount of RuBisCO synthesized. Studies with other facultative autotrophs have shown that the growth of these organisms in the presence of organic

substrates results in the repression of RuBisCO (Kornberg et al., 1960).

In recent years, some observations have been made on the molecular regulation of CO_2 assimilation and regulation of RuBisCO in Rhodospirillum rubrum. a purple non-sulphur bacterium. It was first shown in 1967 by Anderson and Fuller that in R. rubrum. RuBisCO is induced over 30-fold in cells grown with molecular hydrogen as the electron donor and CO_2 as the sole carbon source, as opposed to photoheterophic growth on malate. Subsequent work by Tabita and McFadden (1974a) demonstrated that R. rubrum grown photoheterotrophically on a reduced substrate such as butyrate results in a derepression in the synthesis of RuBisCO to a level of 8% of the total soluble protein. In their study, it was found that external CO_2 in the form of HCO_3 was needed for growth of R. rubrum and that RuBisCO was synthesized only after active growth ceased in the butyrate-HCO_3 medium (Tabita, 1981). It was also observed that significant amounts of RuBisCO was synthesized when R. rubrum was grown on highly reduced fatty acids, though these growth substrates became increasingly toxic with increase in chain length (Tabita, 1981).

In 1979 Schloss and co-workers (Schloss et al., 1979) showed that after a period of 10-14 weeks adaptation to H_2 and CO_2 growth, the RuBisCO in R. rubrum comprised up to 40% of the soluble protein, a level similar to those found in the chloroplast of eukaryotic cells. It is not clear whether the extra-ordinarily high levels of enzyme obtained represent the natural selection of a mutant during this rather lengthy period or whether some form of adaptation to the growth condition occurs with the concurrent overproduction of RuBisCO. However, Sarles and Tabita (1983) showed that when R. rubrum was grown photolithotrophically in an

atmosphere containing low levels of CO_2 (1.5 to 2%) enzyme synthesis was derepressed, with the results that the enzyme comprised up to 50% of the soluble protein of the cells, as determined by immunological methods. Studies with R. rubrum so far, show no indication that either activation or inactivation of the preformed enzyme is an important mode of control, rather the presence of excess CO_2 appears to signal a repression in the synthesis of RuBisCO in this organism.

In the phototrophic bacterium Rhodopseudomonas palustris, Eley et al. (1979) showed that RuBisCO activity present under photolithotrophic growth conditions was repressed by chemoheterotrophic growth, but was not decreased by the presence of organic substrates during photoheterotrophic growth. However, Eley and co-workers did not demonstrate any derepression of RuBisCO in cells grown under photolithotrophic conditions At the moment there is still very little information as regards the regulation of RuBisCO in other Rhodospirillaceae.

In contrast to the investigations dealing with the autotrophic growth of the Rhodospirillaceae (purple non-sulphur bacteria) such as R. rubrum. relatively few systematic studies have been carried out concerning the regulation of RuBisCO in the Chromatiaceae (purple sulphur photosynthetic bacteria). Nevertheless, it has been reported that in Chromatium sp. the activity of RuBisCO was increased under autotrophic growth conditions (Akazawa, 1979). However, the mechanism of regulation of enzyme synthesis and the induction of the Calvin-Benson cycle enzymes in these bacterial cells is not known. Kobayashi and Akazawa (1982) demonstrated that in Chromatium vinosum, repression of RuBisCO was caused by CO_2 concentrations higher than 1 mM. The level of these enzymes was also found to be very low under heterotrophic growth on pyruvate, and

autotrophic growth in the presence of oxygen.

The control of the synthesis of RuBisCO in the chemosynthetic bacteria appears to be quite different from that in photosynthetic bacteria. This concerns primarily the response to exogenous concentrations of CO_2. By contrast to the findings of Tabita (1981) as regards expression of RuBisCO in R. rubrum mediated by butyrate-HCO_3 medium, Beudeker et al. (1980) showed that there is an inverse correlation between the RuBisCO activity and the CO_2 concentration in the chemostat grown cultures of Thiobacillus neopolitanus. Moreover, the fluctuation of the total and particulate RuBisCO activity correlated with changes in the volume density of carboxysomes in the cell, intracellular polyhedral bodies known to contain this enzyme in Thiobacillus neopolitanus (Shively et al., 1973) and Anabaena cylindrica (Codd and Stewart, 1976), and Nitrobacter agilis (Shiveley et al., 1977). The presence of RuBisCO in carboxysomes and in the cytosol of these organisms may suggest a role of carboxysomes in the regulation of RuBisCO synthesis (Codd and Marsden, 1984). The possible role of carboxysomes in the regulation of Calvin cycle and CO_2 fixation has been considered previously (Section 1.2.1.4.3). A detailed study of the molecular basis for the apparent differences between photosynthetic and chemosynthetic organisms should go a long way towards the understanding of the regulation of CO_2 assimilation.

In contrast to obligate chemolithotrophs which are known to be unable to repress the enzymes of the Calvin cycle in the presence of organic compounds (Taylor and Hoare, 1969; Kuenen and Veldkamp, 1973), the facultative chemolithotrophs repress RuBisCO in the presence of heterotrophic substrates as demonstrated in Alcaligenes sutrophus

(Friedrich et al., 1981; Friedrich, 1982). It has also been shown in A. eutrophus (Friedrich, 1982) that RuBisCO is derepressed in cells grown autotrophically in CO_2 limited chemostats.

From this survey, it is apparent that the regulation of RuBisCO is mediated by different mechanisms which depend on the group of organisms under consideration. Moreover, little information is available concerning the mechanism of regulation of synthesis of this enzyme from the purple non-sulphur bacteria (Rhodospirillaceae). In view of the fact that these organisms are probably more versatile than many other autotrophs, and the relative ease by which they may be genetically manipulated (McFadden, 1973; Saunders, 1978), a detailed study of the regulation of synthesis of RuBisCO in the Rhodospirillaceae with a view to understanding more of its properties which are of significance to agriculture is worthwhile.

1.6 Glycollate Metabolism

In organisms fixing CO_2 via ribulose bisphosphate carboxylase as the primary carboxylating enzyme, glycollate formation is a common characteristic. The oxygenase activity of RuBP carboxylase produces 2-phosphoglycollate which is hydrolysed by a specific phosphatase to give glycollate (Richardson and Tolbert, 1961). Several theories concerning the source of photorespiratory glycollate have been proposed. The most prominent of these are: i) oxidation of thiamine pyrophosphate-glycolaldehyde, an intermediate formed during photosynthetic transketolase reaction by oxygen (Coombs and Whittingham, 1966); ii) direct reductive condensation of two molecules of CO_2 and a non-specific

carboxylation reaction (Zelitch, 1964). No substantial role however, has been ascribed to these mechanisms and the only significant source of photorespiratory glycollate *in vivo* is the hydrolysis of 2-phosphoglycollate (the product of the RuBP oxygenase reaction).

Glycollate synthesis and excretion has been shown to occur in eukaryotic algae (Colman *et al*., 1974) in cyanobacteria (Cheng *et al*., 1972), anaerobic photosynthetic bacteria when exposed to oxygen (Codd and Smith, 1974; Codd and Turnbull, 1975) and in some facultative chemolitotrophs (Codd *et al*., 1976; King and Anderson, 1980).

The metabolism of glycollate has been well studied in higher plants and algae, but less work has been done with prokaryotic systems. In higher plants and algae metabolism of glycollate proceeds via glyoxylate-glycine-serine-glycerate (the so called photorespiratory carbon oxidation pathway) to yield 3-phosphoglycerate, which is returned to the Calvin cycle (Ogren, 1984). Cyanobacteria presumably use a different route also leading to the formation of 3-phosphoglycerate, the glycerate pathway involving glyoxylate -tatronic-semialdehyde-glycerate as intermediates (Codd and Stewart, 1973). This pathway is also thought to occur in *Pseudomonas* species when growing on C-2 compounds such as glycollate or glyoxylate (Kornberg, 1966) and in *Alcaligenes eutrophus* (Friedrich *et al*., 1979). These pathways are summarized in Figur 1.13. The reasons for the excretion of glycollate in some prokaryotes is not clear, neither is it well understood whether glycollate can be re-absorbed and metabolised as in the plant system.

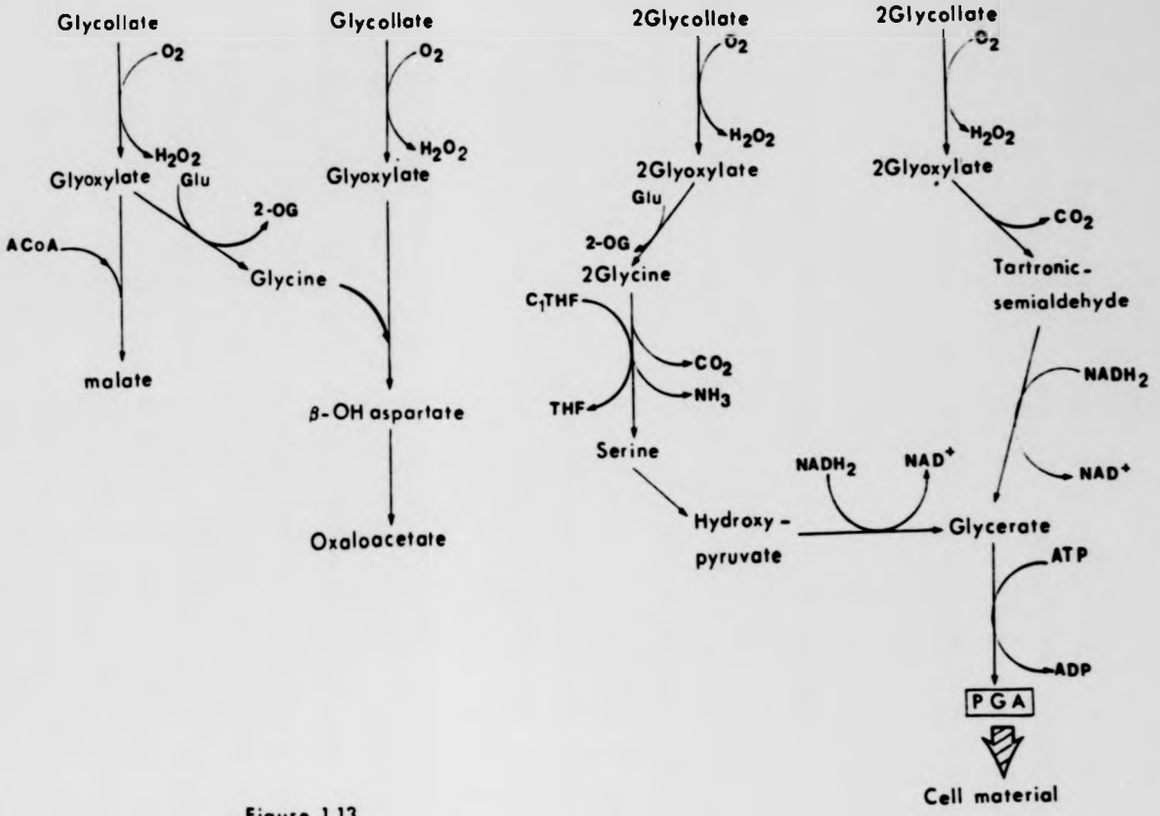


Figure 1.13

Pathways of glycollate metabolism

1.7 Genetics of RuBisCO - and its application to Agriculture

One of the properties of ribulose bisphosphate carboxylase/oxygenase which has been considered to be the most important metabolic constraint on plant productivity is its oxygenase activity since this results in photorespiration (Hardy et al., 1978). Improving the efficiency by which carbon dioxide competes with oxygen for reaction with ribulose bisphosphate may improve the rate of photosynthesis relative to that of photorespiration and could substantially increase productivity in a large number of commercially important crops. This prediction has, in recent years, stimulated considerable research interest on the genetic reduction of photorespiration, which it is hoped will result in an increased rate of carboxylation.

There are three possible changes in RuBisCO by which the rate of carboxylation could be increased and these are: i) by increasing the levels of RuBisCO within the cells; ii) by increasing the affinity of the enzyme for carbon dioxide, and iii) by increasing the ratio of carboxylation to oxygenation. The first change required alteration in the system that controls the levels of this enzyme within the cell. The other two changes involve alterations in the primary structure of the RuBisCO subunit(s) that are involved in catalysis.

Two main lines of approach are being directed to solving these problems. One of these is the conventional biochemical approach to altering the carboxylation/oxygenation ratio in favour of the carboxylase. Support for this view is the fact that yields of crops such as grain legumes can be increased 50-100% by raising the concentration of CO_2 five-fold above the atmospheric concentration (Hardy et al., 1978). This method, though

plausible, is of little practical application.

The second main line of approach is the genetic reduction of photorespiration in plant species. This has been considered as an attractive strategy since, in principle, it involves the modification of only one gene - the gene that encodes the catalytic large subunits of RuBisCO (Somerville and Ogren, 1982). In higher plants and algae attempts have been made to produce altered forms of RuBisCO by mutagenesis of cells but no success in reducing photorespiration by this means has yet been achieved (see Reviews by Sommerville and Ogren, 1982; Ogren *et al*., 1984). The techniques of *in vitro* genetic manipulation to alter the primary sequences by site-directed mutagenesis in attempts to alter the kinetic properties of the enzyme are now being actively pursued in various laboratories. To this end, the genes coding for the large subunits of RuBisCO from a number of plant species have been cloned into *E. coli* (McIntosh *et al*., 1980; Zurawki *et al*., 1981; Gatenby, 1984). These results thus open a new way to *in vitro* genetic manipulation of the RuBisCO enzyme.

Recently the genes coding for the large subunits of RuBisCO from a number of photosynthetic prokaryotes have been cloned. These include the large subunits of the *R. rubrum* enzyme (Somerville and Somerville, 1984), the Form II enzyme from *Rhodopseudomonas sphaeroides* (Fornari and Kaplan, 1983; Quivey and Tabita, 1984; Muller *et al*., 1985). Of interest is the finding that the cloned genes when expressed in *E. coli* synthesise catalytically active RuBisCO (Somerville and Somerville, 1984; Quivey and Tabita, 1984). Of similar interest is the work of Gutteride and Lorimer (see Barber, 1984) on the *in vivo* site-directed mutagenesis of the RuBisCO enzyme of *R. rubrum*. Gutteridge and Lorimer chose to replace

the highly conserved aspartic acid residues 198 by glutamic acid. The modified enzyme, synthesised in E. coli. was found to have lower carboxylase and oxygenase activity as well as a modification in its divalent cation binding properties (Barber, 1984).

Now that it is possible to synthesise active RuBisCO in vitro (Chory et al.. 1985), it seems highly likely that the powerful technique of site directed mutagenesis will soon be used to genetically alter the enzyme to reduce its oxygenase activity and to increase the efficiency of its carboxylation of RuBP. It remains to be seen, however, whether the overall efficiency of photosynthesis in intact plant will thereby be increased, thus their productivity, or whether photorespiration turns out to be obligatory for effective carbon assimilation by the Calvin cycle pathway in C_3 plants. It is almost certain that the genetic approaches to these problems will be best studied using the photosynthetic prokaryotes.

MATERIALS AND METHODS

2.1 Organisms

Rhodopseudomonas blastica strain (NCIB 11576) isolated by Eckersley and Dow (1980), Rhodopseudomonas sphaeroides strain (NCIB 8253), Rhodopseudomonas palustris strain (NCIB 8288) and Rhodospirillum rubrum strain (NCIB 8255) were all from the University of Warwick culture collection. Rhodomicrobium vannielii strain (RM5), previously isolated and characterised by Dr. C. S. Dow (University of Warwick) was used in this study. Pseudomonas oxalaticus OX1 was obtained from Dr. L. Dijkhuizen (University of Groningen, The Netherlands).

2.2 Chemicals and Gases

Acrylamide, N,N'-methylene bisacrylamide and N,N,N',N'-tetramethyl ethylenediamine were purchased from the Eastman Kodak Co., Kirkby, Liverpool, U.K. Ribulose bisphosphate tetrasodium salt and trypsin were supplied by Sigma Chemical Co. Ltd., Poole, Dorset, U.K. Unless otherwise stated, all other chemicals were obtained from the following manufacturers: British Drug Houses (BDH) Ltd., Poole, Dorset; Fisons Scientific Apparatus Ltd., Loughborough, Leicestershire; Hopkin and Williams Ltd., Chadwell Heath, Essex.

Oxygen free nitrogen was obtained from the British Oxygen Co. Ltd., London. Radiochemicals were purchased from the Amersham International plc, Amersham, Buckinghamshire.

2.3 Buffers

TEMMB Buffer

20 mM	Tris-HCl, pH 8.0
10 mM	$MgCl_2.6H_2O$
50 mM	$NaHCO_3$
1 mM	EDTA
5 mM	2-mercaptoethanol

TMM Buffer

20 mM	Tris-HCl, pH 8.0
5 mM	$MgCl_2.6H_2O$
5 mM	2-mercaptoethanol

TE Buffer

20 mM	Tris-HCl, pH 8.0
5 mM	EDTA

Ribulose bisphosphate carboxylase oxygenase (RuBisCo) assay buffer

100 mM	Tris-HCl, pH 8.0
16 mM	$MgCl_2.6H_2O$

Other buffers used in this work are specified in the text.

2.4 Media

2.4.1 Batch Cultures

Species of Rhodospirillaceae were routinely grown on a medium of the following ($g\,l^{-1}$ of distilled water):

NH_4Cl	0.5
$MgSO_4.7H_2O$	0.4
NaCl	0.4
$CaCl_2.2H_2O$	0.05
Sodium hydrogen malate	1.0
Sodium pyruvate	1.0
Yeast extract	0.5

The medium was adjusted to pH 6.8 with potassium hydroxide, and then autoclaved. After cooling, 50 ml l^{-1} of sterile phosphate buffer (0.1 M, pH 7.0) was aseptically added giving a final concentration of 5 mM.

For studies of substrate utilization, sodium hydrogen malate, sodium pyruvate and yeast extract in the basal salt medium were replaced by nicotinic acid(0.1 mg); thiamine hydrochloride (1.0 mg); biotin (0.02 mg); trace elements solution (Pfennig and Lippert, 1966), 1 ml, and the test substrate (added to a final concentration of 10 mM). When necessary, carbon dioxide was added as filter sterilised sodium bicarbonate to give a final concentration of 5 mM.

2.4.2 Continuous Culture

Rhodopseudomonas blastica was grown in continuous culture on medium containing the following (g l^{-1} distilled water):

NH_4Cl	1.0
$MgSO_4.7H_2O$	0.5
$CaCl_2.2H_2O$	0.1
NaCl	0.5
Sodium hydrogen malate	1.0
adjusted to pH 4.5	

The medium routinely contained (per litre mineral base): thiamine hydrochloride 250 µg; nicotinic acid 0.1 mg; biotin 250 mg; trace elements (Pfennig and Lippert, 1966) 1 ml and 0.001% yeast extract. After autoclaving and cooling, 0.1 M phosphate buffer, pH 7.0 was aseptically added to give a final concentration of 5 mM.

For carbon dioxide limited continuous culture, two reservoir pots were used, one containing the above medium from which sodium hydrogen malate was omitted, and the other contained sodium butyrate (10 mM) and filter sterilised $NaHCO_3$ (2.5 mM final concentration at pH 12.0).

For light limited chemostat culture, the medium containing 1.5 gl^{-1} of sodium hydrogen malate was used.

2.4.3 Solid Media

When solid medium was required 1.5%(w/v) agar was added.

2.5 Maintenance of Cultures

Members of the Rhodospirillaceae used in this study were regularly subcultured and maintained on pyruvate-malate medium as agar stabs and agar plates. These were then incubated in anaerobic bags by the method of Westmacott and Primrose (1975), as modified by Dow and France (1980), at 30°C with an incident light intensity of 2000 lux from tungsten lamps. After growth, the agar stabs and plates were maintained at room temperature in the light. Stock cultures were stored in sterile 90% (v/v) glycerol at -20°C.

2.6 Culture Purity

The purity of all cultures was checked by streaking onto nutrient agar plates and incubating such plates both anaerobically at a light intensity of 2000 lux, and aerobically at 30°C in the dark. Plates were examined with an Olympus Model X-Tr stereoscopic plate microscope. Cultures were also examined by high power phase contrast microscopy.

2.7 Spectrophotometry

Measurement of culture absorbances at 650 nm (A_{650}) and protein determinations by Lowry's and Bio-Rad methods were performed on a Pye Unicam SP500 spectrophotometer. All spectrophotometric enzyme assays were done using a Pye Unicam SP1800 recording spectrophotometer fitted with a constant temperature cuvette housing and linked to a Unicam AR25 linear recorder.

2.8 Growth of Organisms

2.8.1 Batch Culture

Members of the Rhodospirillaceae were routinely grown in 250 ml Erlenmeyer flasks, containing 100 ml of medium, which were sealed with rubber serum caps (William Freeman & Co. Ltd., Barnsley, Yorkshire). Each flask was gassed with oxygen free nitrogen via 'inlet' and 'outlet' syringe needles inserted through the serum cap. The flasks were then incubated on an orbital shaker (L.H. Engineering, Stoke Poges, Bucks.) at 30°C under constant illumination from tungsten lamps (incident light intensity of 2000 lux). Large cultures were grown in 20 L pots fitted with a Quickfit MAF 2/2 lid (Corning Ltd., Stone, Staffs.). After flushing with oxygen free nitrogen, these were incubated at 30°C with constant stirring via a magnetic follower and an incident light intensity of 2000 lux from tungsten lamps. Cells for enzyme purification, were harvested during the early stationary phase of growth (A_{650} = 1.8 to 2.0).

The effect of growth substrates on the activity of ribulose bisphosphate carboxylase/oxygenase from R. blastica was determined using cultures of 150 ml final volume in 250 ml Erlenmeyer flasks prepared as described above. However, in this case, cells were harvested during the mid-exponential and the early-stationary phases of growth.

2.8.2 Continuous Cultures

R. blastica was grown in continuous culture under different growth conditions. Unless otherwise stated, the cultures were maintained at pH

7.0 by titrating with either 1 M NaOH or 1.0 M HCl, and growth was followed by sampling and measuring the absorbance at 650 nm. Steady states were reached when the absorbance, pH and the temperature of the cultures remained constant for a period of time, usually after five culture volume changes. At steady state, cells were harvested and drop frozen in liquid nitrogen and stored at -20°C until required.

2.8.2.1 Chemoheterotrophic growth

R. blastica was grown chemoheterotrophically on malate under aerobic conditions in the dark in a 1.5 L fermenter (New Brunswick Scientific Co., New Brunswick, New Jersey) with a working volume of 900 ml. R. blastica cells were grown at different dilution rates ranging from 0.001 to 0.15 h^{-1}. Such cultures were constantly stirred and aerated at a flow rate of 500 ml min^{-1}. The oxygen partial pressure in the cultures was determined using an oxygen electrode (Yellow Spring Instrument Co., Yellow Springs, Ohio). Temperature was maintained at 30°C by a water jacket connected to a thermocirculator (Churchill Instrument Co. Ltd., Perivale, Middlesex).

2.8.2.2 Aerobic growth in the light

R. blastica was grown aerobically in the light (malate as carbon source) in a chemostat similar to the one described for chemoheterotrophic growth, except that the culture was illuminated by tungsten bulbs giving an incident light intensity of 10,000 lux. Temperature was maintained at 30°C by a circulating water jacket connected to a thermocirculator.

2.8.2.3 Photoheterotrophic Growth

R. blastica was grown photoheterotrophically on malate in a 1 L chemostat having a working volume of 750 ml. Anaerobic conditions were maintained by flushing the culture, reservoir medium, and all the connecting tubes with oxygen free nitrogen. The culture was stirred constantly and illuminated at 10,000 lux. Temperature and pH were maintained at 30°C and 7.0 respectively. Cells were grown at a dilution rate of 0.05 h^{-1}.

2.8.2.4 Light Limited Growth

Light limited cultures of R. blastica were maintained in a chemostat similar to the one described for photoheterotrophic growth except that an incident light intensity of 1500 lux was used. Under these conditions, the chemostat study was carried out in a darkened room where there was no alternative source of light other than that used to illuminate the culture. A dilution rate of 0.05 h^{-1} was used.

2.8.2.5 Carbon dioxide - Limited Growth

CO_2 limited culture of R. blastica was achieved on butyrate-HCO_3 medium in a 1 L fermenter. Cells were grown at a dilution rate of 0.05 h^{-1} and the incident light intensity was 10,000 lux. Anaerobic condition was maintained under oxygen free nitrogen.

2.9 Total Carbon and Inorganic Carbon Estimation

The total carbon and inorganic carbon in the culture supernatant were determined with a carbon analyzer (Beckman model 915A), connected to an

infra-red analyzer (Beckman model 865). Culture supernatant was prepared by filtration of 2 ml samples through a 0.2 µm Millipore filter. The total carbon in the culture supernatant was estimated by injecting 50 µl of the sample into the total carbon channel of the carbon analyzer which had been calibrated at 1000°C. The concentration of the total carbon was determined from a calibration curve obtained with known concentrations of potassium biphthalate. The dissolved inorganic carbon was determined by injecting 50 µl of the culture supernatant into the inorganic channel of the carbon analyzer at 300°C. The concentration of inorganic carbon was then determined from standard curves obtained using sodium bicarbonate dissolved in CO_2-free water. The difference between the total carbon and inorganic carbon was taken as a measure of the organic carbon in the culture supernatant.

2.10 Bacterial Dry Weight Estimation

Bacterial dry weight was determined by filtering 3.0 ml of the culture through dried and pre-weighed 0.2 µm membrane filters (Oxoid Ltd., London). These were then dried at 60°C to constant weight after cooling in a desicator. In some instances the bacterial dry weight was determined as the total carbon as described by Dijkhuizen (1979).

2.11 Cell Harvesting and Storage

Small cultures were harvested by centrifugation at 10000 x g for 30 min in a 6x300 ml aluminium angle head rotor at 4°C. Large cultures were

harvested at 4°C either in a continuous head rotor at 14000 x g or by filtration in a Millipore Ultrafiltration Unit (Millipore Ltd., Harrow, Middlesex). Cell pellets were washed twice with TEMMB buffer and resuspended in TEMMB buffer. When appropriate cells were drop frozen in liquid nitrogen and stored at -20°C until required.

2.12 Preparation of Soluble Extracts

All steps were carried out at 0°-4°C. Cells were broken by two passages of the cell suspension through a pre-cooled French Pressure Cell (American Instrument Co., Maryland) at 137 MPa. In several instances, 2 ml aliquots of the cell suspension were sonicated at 20 KHz for 20 sec at 0°C with a MSE Ultrasonic disintegrator fitted with a 2 mm probe (MSE Ltd., Crawley, Sussex). This procedure was repeated several times with 1 min cooling intervals until maximum cell breakage was achieved. Unbroken cells and debris were removed by centrifugation at 10,000 x g for 10 min (30 min for *R. blastica*). The supernatant was subjected to high speed centrifugation at 120,000 x g for 1.5 h to yield the soluble fraction.

Extracts of *Pseudomonas oxalaticus* were prepared by the method of Dijkhuizen *et al.* (1978). Cells were resuspended in 50 mM potassium phosphate buffer, pH 7.5. After cell breakage the mixture was centrifuged at 20,000 x g for 1 h. The supernatant was used as the soluble fraction.

2.13 Measurement of CO_2 Fixation by Intact Cells

The incorporation of $^{14}CO_2$ by intact cells of R. blastica was measured with cell suspensions by the modified method of Dijkhuizen et al. (1978). Cells were harvested by centrifugation in a bench centrifuge at room temperature, washed and resuspended in 20 mM Tris-HCl, pH 7.5 (1.0 mg of dry weight of cells ml^{-1}) (Dijkhuizen et al., 1978). The reaction mixture (0.25 ml) contained test substrate, 8 µmol in, Tris-HCl buffer, pH 7.5, and 0.1 mg dry weight of bacteria. The reaction was started after preincubation for 5 min at 30°C by the addition of [^{14}C]-sodium bicarbonate (1.2 µmol, 1 µCi $mmol^{-1}$). The reaction mixture was flushed with oxygen free nitrogen and the tubes illuminated at 2000 lux. The rate of $^{14}CO_2$ incorporation was followed for 30 min by stopping the reaction in a series of test tubes at 1 min intervals by dilution with 1 ml Tris-HCl, pH 7.5, buffer containing 120 µmol of cold bicarbonate. Cells were harvested by filtration through a 0.2 µm Millipore filter. The filters were dried at 100°C for 20 min, cooled and 5 ml of scintillation fluid added. Radioactivity was then counted in a liquid scintillation counter (Packard, Tri-carb Model 3320).

2.14 Measurement of CO_2 Fixation by Cell Free Extracts

Carbon dioxide fixation by cell free extract was measured in a reaction mixture (0.25 ml final volume) which contained 15 µmol Tris-HCl, pH 8.2, 2.5 µmol $MgCL_2.6H_2O$, 5 µmol $NaH^{14}CO_3$ (specific activity 0.8 µCi μmol^{-1} unless otherwise stated), soluble extract 50 µl, and when required ATP and AMP (0.2 µmol of each). After preincubation for 5 min at 30°C, the

reaction was started by the addition of 0.2 µmol of the test substrate. The reaction was stopped after 2 min by the addition of 100 µl of 12 M formic acid. Any precipitate was removed by centrifugation for 5 min in a microfuge. A 200 µl supernatant sample was removed and evaporated to dryness in a scintillation vial. The dried sample was resuspended in 200 µl water and 3 ml of scintillation fluid added. The stable radioactive product was counted in a scintillation counter. Control assays simultaneously lacked any test substrate.

2.15 Enzyme Assays

All enzyme assays were done at 30°C.

2.15.1 RuBP carboxylase

RuBP carboxylase [3-phospho-D-glycerate carboxylyase (dimerizing); EC4.1.1.39] was assayed by the RuBP-dependent incorporation of $NaH^{14}CO_3$ into acid stable products by the method described by Taylor and Dow (1980). The reaction mixture (0.27 ml final volume) contained 150 µl of RuBP carboxylase assay buffer (100 mM Tris-HCl, pH 8.2 and 16.0 mM $MgCL_2.6H_2O$), 5 µmol $NaH^{14}CO_3$ (0.8 µCi μmol^{-1}) and the enzyme solution. The reaction mixture was incubated at 30°C for 5 min after which 50 µl of 5 mM RuBP was added to start the reaction. After 2 min, the reaction was stopped by the addition of 100 µl of 12 M formic acid. A 200 µl sample was removed and evaporated to dryness. Radioactivity was counted as described above.

For pH studies, assays were performed using the procedure except that the incubation mixture contained the following buffering species: Tris-HCl at pH 7.0, 7.2, 7.4, 7.6, 7.8, 8.0, 8.4 and 8.6.

Metal ion requirement and inhibition studies were performed using the RuBP carboxylase assay system. Such metal ions or the inhibitors were added to the reaction mixture before the initation of the reaction with RuBP.

2.15.2 RuBP oxygenase

This was assayed by the method described by Taylor (1979).

2.15.3 Phosphoribulokinase

Phosphoribulokinase activity was measured in a reaction coupled to the RuBP carboxylase assay. Assays were performed using the standard procedure for RuBP carboxylase except that ATP (0.2 µmol) was included in the reaction mixture and ribulose bisphosphate omitted. Ribulose-5-phosphate was used to initiate the reaction. The RuBP produced by the action of phosphoribulokinase was then assayed for by using the purified form I ribulose bisphosphate carboxylase/oxygenase from R. blastica.

2.15.4 Enzyme unit

One unit of activity is defined as the amount of enzyme required to transform 1 µmol of substrate per min.

2.16 Protein Determination

The protein concentration in the soluble extracts was determined by the method of Lowry *et al.* (1951). In several instances, the Bio-Rad protein dye reagent was used according to the manufacturers instructions for the determination of proteins in solution. Dried crystalline bovine serum albumin was used as standard.

2.17 Partial Purification of RuBisCo by Sucrose Gradient Centrifugation

A one-step isolation procedure was used for partial purification of RuBisCo from a number of the *Rhodospirillaceae*. In this study, sucrose solutions were prepared in TEMMB buffer at concentrations ranging from 0.2 to 0.8 M. Linear (0.2 to 0.8 M) gradient or step sucrose gradients of equal volumes of 0.2, 0.3, 0.4, 0.5, 0.6, 0.7 and 0.8 M were routinely prepared in 25 ml polycarbonate centrifuge tubes. The final volume of the gradients did not exceed 15 ml. Soluble extracts (2 ml; up to 40 mg protein) were loaded onto the gradients and then topped with liquid paraffin. The gradients were centrifuged at 240,000 x g for 2½ h in a pre-cooled 8x25 Titanium angle rotor at 4°C in a MSE 65 superspeed centrifuge. After centrifugation, the gradient was left in the vertical position for 10 min before it was harvested from the bottom. Fractions (1 ml each) were collected and monitored for protein at 280 nm. Each fraction was then assayed for RuBP dependent $^{14}CO_2$ fixation. Active fractions were pooled and concentrated by ultrafiltration in an Amicon ultrafiltration unit fitted with a PM10 membrane (mol. vol. cut off-

1000). The pooled RuBisCo fractions were run on non-denaturing polyacrylamide gels (see section 2.20.2) and analysed by the procedure described below.

2.18 Polyacrylamide Gel Assay for Ribulosebisphosphate Carboxylase

To locate enzymic activity in polyacrylamide tube gels, an *in situ* polyacrylamide gel assay for RuBP carboxylase was developed. For these experiments, the sample to be analysed was electrophoresed on two duplicate non-denaturing tube gels, one of which was stained for protein and destained in the usual manner (section 2.20.4). The other was frozen at -20°C and then sliced into 1 mm slices with a gel slicer. Each of the slices was placed in a 1 ml eppendorf tube containing the constituents of the RuBisCo assay in a total volume of 0.25 ml. The tube was kept at 2°C with gentle shaking for at least 3 h in order to elute the protein from the gel slice into the reaction mixture. This was then incubated at 30°C for 10 min and RuBP added to initiate the reaction. The assay was allowed to proceed for at least 15 min in order to detect low levels of activity. At the appropriate time, the assay was terminated by the addition of 100 µl of formic acid. A 200 µl sample of the acid stable product was removed and evaporated to dryness in a glass minivial at 105°C. The dried sample was resuspended in water and scintillation fluid added and the radioactivity determined.

2.19 Purification of RuBisCo from R. blastica

Frozen cell paste was thawed and resuspended in TEMMB buffer (1 g ml^{-1} of buffer). The cell suspension was twice passed through a pre-chilled French Pressure Cell at 137 MPa. Unbroken cells and cell debris were removed by centrifugation at 10,000 x g for 30 min and the supernatant decanted. The pellet was washed twice with TEMMB buffer, centrifuged as before and the supernatants combined. The pooled supernatants were then subjected to ultracentrifugation at 120,000 x g for 60 min at 4°C (Beckman L65 ultracentrifuge using a type 8x25 Aluminium rotor) to remove membrane. All steps were done at 0-4°C except where indicated. A 1 M solution of $MgCl_2.6H_2O$ was added to the supernatant to give a final concentration of 50 mM. The extract was then immersed in a water bath at 50°C for 10 min (Gibson and Tabita, 1977a, b), after which it was rapidly cooled in an ice-bath. The turbid suspension was centrifuged at 10,000 x g for 30 min to remove denatured protein and chromatophore membranes.

The supernatant was then taken to 60% (w/v) ammonium sulphate saturation, stirred for 30 min and allowed to stand for 60 min at 4°C. The suspension was centrifuged at 40,000 x g for 30 min and the supernatant decanted. The pellet was dissolved in minimum amount of TEMMB buffer and dialysed overnight against TEMMB buffer.

The dialysed enzyme solution was centrifuged at 40,000 x g for 30 min to remove any denatured protein, and the supernatant loaded onto a Bio-gel A-5M column (3.5 x 100 cm) equilibrated with TEMMB buffer. Protein was then eluted from the column with TEMMB buffer at a flow rate of 13.0 ml

h^{-1}, and 5 ml fractions were collected using an LKB Ultrorac Fraction Collector. Active fractions were pooled and concentrated in an Amicon ultrafiltration apparatus fitted with a PM10 membrane.

The concentrated enzyme solution was then applied to a DEAE trisacryl column (2 x 10 cm) equilibrated with TEMMB buffer. Three times the column volume of TEMMB buffer was passed through the column. These washings contained no RuBP dependent CO_2 fixing activity. A linear gradient of 0.01 M to 0.2 M NaCl in TEMMB buffer was passed through the column at a flow rate of 10 ml h^{-1} and 5 ml fractions collected. Two peaks of RuBP dependent CO_2 fixing activity eluted from the column.

Active fractions of each peak were pooled separately and concentrated by ultrafiltration in an Amicon ultrafiltration unit fitted with a PM10 membrane. The concentrated enzyme solution was finally loaded onto a step sucrose gradient comprising of seven successive layers (2 ml each) of 0.8, 0.7, 0.6, 0.5, 0.4, 0.3 and 0.2 M sucrose in TEMMB buffer. The gradient was then centrifuged at 240,000 x g for 2½ h in an MSE 65 superspeed ultracentrifuge using an 8 x 25 Titanium angle head rotor. The gradient was harvested from the bottom and 1 ml fractions collected. Active fractions were pooled and concentrated by ultrafiltration.

2.20 Polyacrylamide Gel Electrophoresis (PAGE)

The analysis of SDS-denatured proteins was carried out on polyacrylamide slab gels while native proteins were analysed on non-denaturing polyacrylamide tube gels.

2.20.1 SDS-polyacrylamide slab gels

a) Stock solutions:

High bis acrylamide

60 g acrylamide

1.6 g bisacrylamide

taken to 100 ml with double distilled water.

Low bis-acrylamide

10 g acrylamide

0.5 g bisacrylamide

taken to 100 ml with double distilled water.

TEMED - used as supplied (stored at 4°C)

Stacking gel acrylamide

10 g acrylamide

0.5 g bisacrylamide

taken to 100 ml with double distilled water.

Ammonium persulphate (10% w/v) was freshly prepared.

Resolving gel buffer

3 M Tris-HCl, pH 8.8

36.33 g Trizma Base

in double distilled water and adjusted to pH 8.8

with concentrated HCl.

Stacking gel buffer

0.5 M Tris-HCl, pH 6.8

6.0 g Trizma base in double distilld water adjusted to pH 6.8 with concentrated HCl and made up to 100 ml.

Running buffer (5 x stock)

30.2 g Trizma base

144 g glycine

L^{-1} double distilled water

Before use, 10 ml of 10% (w/v) SDS was added to 200 ml of the stock solution and then made up to 1 L with double distilled water.

b) Preparation of SDS polyacrylamide slab gels

Single percentage polyacrylamide gel

Clean slab gel plates (20 cm x 20 cm) were held the correct distance apart with vaseline greased Teflon spacers (1.5 mm thick). The bottom of the glass plates and the outer edge of the spacers were sealed with lightly greased silicon rubber tubing and the assembly clamped together with metal clips. The resolving polyacrylamide gel mixture was prepared by adding the correct volumes of all of the components, excluding TEMED and ammonium persulphate (Table 2.1), and degassed. After degassing, TEMED TEMED and ammonium persulphate were added, and gently mixed by swirling. The acrylamide solution ws then carefully pumped into the space between the plates, leaving sufficient space for the stacking gel. Water-saturated butanol was layered onto the surface of the gel which was

Table 2.1 Recipe for the preparation of SDS-polyacrylamide slab gels

Stock solution (ml)	stacking gel	Resolving gel Concentration % (w/v)	
		10	30
Stacking gel acrylamide	3.0	-	
Stacking gel buffer	2.4	-	
High bisacrylamide	-	8.3	-
Low bisacrylamide	-	-	10.0
Resolving gel buffer	-	6.25	2.5
Glycerol (75% v/v)	-	-	7.3
Water (double distilled)	4.4	34.9	-
SDS	0.1	0.5	0.1
TEMED+	5ul	10ul	4ul
Ammonium persulphate+	0.1	0.1	0.04

+ These were added after the gel solution had been degassed.

subsequently left to polymerise. After polymerization, as evident from the clear interface between the gel and overlay, the overlay was poured away and the surface of the gel rinsed with double distilled water. The stacking gel was prepared according to the protocol in Table 2.1, poured into the remaining space between the plates and the sample well comb immediately inserted. After polymerization, the comb was removed and the sample wells were rinsed and filled with running buffer.

Exponential Gradient Gels

Exponential gradient of 10-30% (w/v) polyacrylamide slab gels were routinely used for the analysis of SDS denatured protein. The slab gel plates (20 x 25 cm) were cleaned and assembled as described for the single percentage gels. The gel mixtures were prepared according to the protocol in Table2.1. After degassing, TEMED and ammonium persuphate were added to the 10% (w/v) and 30% (w/v) acrylamide solutions with gentle mixing. The 10% (w/v) gel mixture was poured into a 20 ml glass vial and sealed with a suba seal having 'inlet' and 'outlet' tubing. The 30% (w/v) gel mixture was then pumped into the 10% (w/v) gel mixture through the 'inlet' tube and the mixture constantly stirred to ensure thorough mixing (figure 2.1). The gel mixture from the 'outlet' tube was poured between the plates to the desired level after which it was allowed to polymerise. The stacking gel was prepared and applied as described above for the single percentage gel.

c) Sample loading and electrophoresis

After polymerization, the gel was mounted in place on the Studier-type electrophoresis apparatus (Hames, 1981). A seal between the glass plate

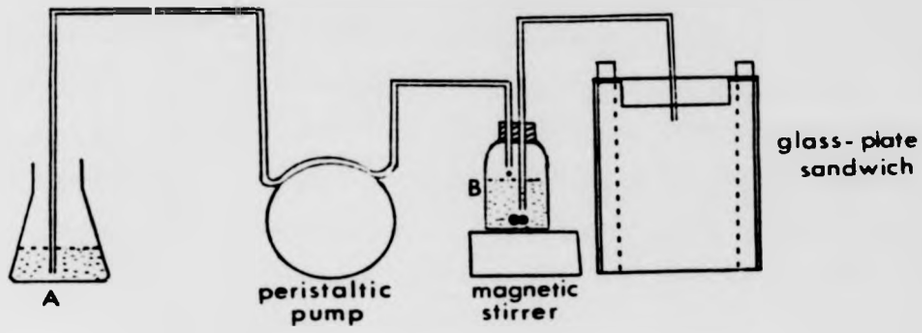


Figure 2.1 A line drawing of the apparatus for the formation of exponential gradient polyacrylamide slab gels. The glass plate is held in position by clamps.

A - contained the low percentage acrylamide.

B - contained the high percentage acrylamide.

and the electrophoresis apparatus was made by coating the area around the apparatus' notch with vaseline before the plate was clamped in position. The upper and the lower reservoir of the electophoresis apparatus were then filled with the running buffer. Any air bubbles at the bottom of the gel were removed. After checking for leaks, the samples were loaded into the sample wells with a microsyringe. Routinely the gels were electrophoresed at 12 mA until the tracking dye just ran off the bottom of the gel. In the case of the 10-30% (w/v) gradient gels, electrophoresis was continued for about 1 h after the tracking dye had run off the gel. After electrophoresis, the gels were stained by one of the methods described in Section 2.20.4.

2.20.2 Non-denaturing gels

A modified method for the analysis of proteins on non-denaturing disc gels (Gabriel, 1971) was used throughout the purification procedures to monitor enzyme purity and also for the analysis of RuBisCo from sucrose gradient isolation procedures.

a) Stock solutions

Stock biscarylamide

32 g acrylamide

0.6 g bisacrylamide

dissolved and made up to 100 ml with double distilled water.

Resolving buffer and TEMED were as for denaturing gels.

Ammonium persulphate (0.14% w/v)

0.14g ammonium persulphate in 100 ml of double distilled water was freshly prepared.

Running buffer

6.0 g Trizma base

28.3 g Glycine

0.75 ml 2-mercaptoethanol

dissolved in double distilled water, adjusted to pH 8.8 and made up to 1 L with double distilled water.

b) Preparation of non-denaturing tube gels

Glass tubes (10 cm x 0.5 cm) were routinely used for the rod gels. Gel tube were thoroughly cleaned, dried and then marked at the required length (usually 8 cm). The tubes were sealed at the bottom with Parafilm and held in position with rubber bands. Having selected the resolving gel concentration to be used, the gel mixture, excluding TEMED and ammonium persulphate, was prepared as indicated in Table 2.2. After degassing for about 5 min, TEMED and ammonium persulphate were added and gently mixed by swirling. Each tube was held in a vertical position by rubber grommets located in the upper buffer reservoir of the electrophoresis apparatus and then filled with the gel solution to the required level using a 2 ml pipette. The gel solution was overlayed with water-saturated butanol and allowed to polymerise.

Table 2.2 Recipe for the preparation of non-denaturing tube gels

Stock solution (ml)	Gel concentration (%)					
	2	3	4	5	6	7
Bisacrylamide Stock*	2.5	3.75	5	6.25	7.5	8.75
Resolving buffer	12.5	11.5	10	8.75	7.5	6.25
Water (double distilled)	5	5	5	5	5	5
TEMED+	0.023	0.023	0.023	0.023	0.023	0.023
Ammonium persuphate+ (0.14%)	20	20	20	20	20	20

* This was prepared as described in Section 2.20.2a

+ Added after degassing

c) Loading and electrophoresis

After polymerization, as evident from the clear interface between the gel and the overlay, the overlay was poured away, and the surface of the gel rinsed with double distilled water. Having assembled the electrophoresis apparatus, the upper and the lower reservoirs were filled with the running buffer and any air bubbles trapped in the space above or below the gel removed. Samples containing up to 45 µg of protein were loaded onto each gel and electrophoresis carried out in the cold room (3°C) at 0.25 mA per gel until the tracking dye could be seen entering the gel. At this point the current was increased to 2.5 mA per gel, and electrophoresis continued until the tracking dye was about 1 cm from the bottom of the gel. The gel was then removed from the tube by water pressure exerted from a 10 ml hypodermic syringe fitted with silicone bung. The gel was either stained for protein or analysed for RuBisCo as described in section 2.17.

2.20.3 Sample preparation

Soluble proteins for SDS-PAGE were dissociated with 0.1% (v/v) SDS in a sample solutin (140 µl final volume) containing 10 µl 2-mercaptoethanol, 10 µl of 75% (w/v) glycerol, 10 µl tracking dye (0.1% w/v bromophenol blue) and 100 µl sample. The mixture was heated at 100°C for 2-3 mins and allowed to cool before loading the appropriate volume onto the gel. Protein standards used for SDS-PAGE were phosphorylase b (94 KDal), bovine serum albumin (67 KDal), ovalbumin (43 KDal), carbonic anhydrase (30 KDal), soya bean trypsin inhibitor (20.1 KDal) and β-lactalbumin (1 4 KDal).

Samples for non-denaturing tube gels were prepared in a similar sample

solution described for SDS-PAGE except that SDS was omitted from the mixture and heat was not applied. When necessary, the protein markers used for non-denaturing gels were thyroglobulin (669 KDal), ferritin (440 KDal) catalase (232 KDal) and aldolase (158 KDal).

2.20.4 Gel staining and recording

The coomassie blue and silver nitrate staining techniques were routinely used to detect protein bands in the gels.

a) Coomassie blue staining

Polyacrylamide gels were stained for 3-4 h (up to 12 h for non-denaturing tube gels) unless otherwise stated, in 0.1% (w/v) coomassie blue AR25 in 45% (v/v) methanol and 10% (v/v) acetic acid. Destaining was done in 45% (v/v) methanol, 10% (v/v) acetic acid for 4 h, followed by overnight soaking in a solution containing 20% (v/v) isopropanol and 10% (v/v) acetic acid. Destaining was completed in a solution of 10% (v/v) isopropanol and 10% (v/v) acetic acid.

b) Silver nitrate staining

The silver nitrate staining technique of Wray *et al.* (1981) was routinely used for the detection of protein bands in SDS-polyacrylamide slab gels. The gel to be stained was first fixed by soaking in 50% (v/v) methanol for at least 6 h with several changes. The staining solution was prepared by dissolving 0.8 g of silver nitrate in 4 ml of distilled water. This solution was then mixed with a solution containing 21 ml of 0.36% (w/v) NaOH and 1.4 ml of 14.8 M ammonia solution, and the volume made up to 100 ml with double distilled water (solution A). The gel was then soaked in solution A for 15 min with

constant gentle shaking after which it was washed several times with deionized water. After washing, the gel was developed by soaking in a solution containing 2.5 ml of 1% (w/v) citric acid and 0.25 ml of 38% (w/v) formaldehyde in a final volume of 500 ml. The reaction was stopped after the protein bands had appeared by washing the gel in double distilled water followed by soaking in 50% (v/v) methanol.

Stained gels were routinely photographed from above using a Pentax SP500 camera with Kodak panatomic X film (ASA 32). In some instances, stained rod gels were scanned for protein at 600 nm using an SP800 spectrophotometric gel scanner connected to a Unicam AR25 linear recorder.

2.21 Holoenzyme Molecular Weight Determination

2.21.1 Gel filtration

The molecular weight of ribulose bisphosphate carboxylase/oxygenase (RuBisCo) from R. blastica was determined at 4°C on a 1 x 90 cm column of Sephadex G200 equilibrated with TEMMB buffer. The standard protein markers used were aldolase (Mr 158,000), catalase (Mr 232,000), ferritin (Mr 440,000) and thyroglobulin (Mr 669,000). A 1.0 ml sample containing the enzyme and protein markers was applied to the column and eluted with TEMMB buffer at a flow rate of 10.0 ml h^{-1}. Two ml fractions were collected. Elution volumes were determined by monitoring the column effluent at 280 nm and, for R. blastica RuBisCo, also by enzymic assay of the fractions. Catalase activity in the fractions was also measured by the method of Chance and Maehly (1955). The void volume of the

column was measured with Blue Dextran 2000.

2.21.2 Non-denaturing PAGE

The holoenzyme molecular weight of RuBisCo was also estimated by non-denaturing polyacrylamide gel electrophoresis by the method of Hedrick and Smith (1968). In this procedure, purified enzyme was run on non-denaturing polyacrylamide gels polymerized from 3, 4, 5, 6 and 7% (w/v) acrylamide as described previously. Molecular weight markers (aldolase, catalase, ferritin and thyroglobulin) were also run in the same manner. By plotting the Rf values of the proteins against gel concentration curves of different slopes were obtained. The molecular weight of the purified RuBisCo was then estimated from the plot of the slopes against Mr of the standards (Hedrick and Smith, 1968).

2.22 Peptide Mapping of the two large Subunits of RuBisCo from R. blastica

Peptide 'finger printing' of the two large subunits of ribulose bisphosphate carboxylase/oxygenase isolated from R. blastica was carried out by a method based on that described by Cleveland et al. (1977). Basically this method involved isolation of the large subunits from the holoenzyme in a one-dimensional SDS polyacrylamide slab gel, digestion of the isolated large subunits and subsequent analysis of the digest by PAGE.

Gel electrophoresis in the first dimension

Purified RuBisCos were run into 10-30% (w/v) exponential gradient

column was measured with Blue Dextran 2000.

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Gel electrophoresis in the first dimension

Purified RuBisCos were run into 10-30% (w/v) exponential gradient

polyacrylamide gels as described in Section 2.20.1. These gels were then stained with a solution containing 0.25% (w/v) coomassie blue AR25, 45% (v/v) methanol and 10% (v/v) acetic acid, and destained by diffusion into a solution of 45% (v/v) methanol, 10% (v/v) acetic acid. To avoid any possible non-specific acid hydrolysis, gels were stained for 15 min and destained for no more than 20 min. Bands of interest were visualized by placing the destained gel on a glass plate over a light box. The individual bands were cut out with a razor blade, trimmed and washed in distilled water, and then soaked in a solution containing 0.125 M Tris-HCl, pH 6.8, 1% (w/v) SDS and 1 mM EDTA (buffer A) for 30 min. At this stage, the gel pieces could be stored frozen at -20°C prior to proteolytic digest. Two methods were routinely used for the digestion of polypeptides and subsequent analysis of the digest peptides.

a) In the first method, the gel slices (after soaking in buffer A) were placed in the wells of a 10-30% (w/v) SDS polyacrylamide slab gel (with a 5 cm stacking gel). The gel pieces were pushed to the bottom of the wells and the spaces around the gel slices filled with buffer A containing 10% (v/v) glycerol. Finally, each slice was overlayed with the appropriate volume of buffer A containing 10% (v/v) glycerol and a given amount of protease. Electrophoresis was then carried out in the usual manner except that the power was switched off for 15 min when the tracking dye neared the bottom of the stacking gel.

b) In the second method, protein from the gel slices was eluted by electrophoresis overnight into a dialysis bag. Eluted proteins were heated at 100°C for 2 min in sample buffer containing 0.125M Tris-HCl,

pH 6.8, 10% (v/v) glycerol and 0.001% (w/v) bromophenol blue. Proteolytic digestions were carried out at 37°C for the appropriate times (as indicated in the figure legends) by the addition of given amounts of proteases. Proteolysis was stopped by boiling for 2 min, and the mixture allowed to cool. The mixtures were then run on a 10-30% (w/v) exponential gradient polyacrylamide gels as described previously. Gels were stained with silver nitrate in order to detect low levels of peptides.

2.23 Time Course of the Expression of Ribulose Bisphosphate Carboxylase/Oxygenase in Rhodopseudomonas blastica

The time course of the expression of RuBisCo in R. blastica was investigated in cells growing photoheterotrophically in batch on pyruvate-malate medium. In this procedure, R. blastica was grown in 5 L pyruvate-malate medium at an incident light intensity of 2000 lux. At appropriate time intervals, 500 ml samples were removed and the pH and optical density at 650 nm measured. The sample was then centrifuged at 10,000 x g for 15 min. The dissolved inorganic carbon in the culture supernatant was determined as described previously. The cell paste was washed and resuspended in a minimum volume of TEMMB buffer. A soluble protein extract was prepared from the cell suspension and this was subjected to the sucrose gradient purification protocol as described previously. Active RuBisCo fractions from the sucrose gradient centrifugation were pooled and analysed for the presence of one or two enzymes by the in situ non-denaturing gel assay system for RuBisCo (Section 2.18).

2.24 [^{35}S]-Methionine Incorporation into Ribulose Bisphosphate Carboxylase/Oxygenase from R. blastica

The synthesis of RuBisCo in R. blastica during growth in batch was investigated by labelling growing cells with [^{35}S]-methionine. R. blastica cells were grown anaerobically in the light (2000 lux) in 500 ml malate or butyrate-HCO_3 medium. At appropriate time intervals, 100 ml samples were removed and transferred into 250 ml Erlenmeyer flasks which were sealed with suba seals and pre-gassed with oxygen-free nitrogen. These were subsequently incubated (with shaking) at 30°C. After 5 min incubation, a 20 ml sample was removed for the estimation of pH, dissolved inorganic carbon in the culture supernatant, and RuBP dependent $^{14}CO_2$ fixation by the soluble protein extract. The remaining cells in the 250 ml Erlenmeyer flask were labelled with [^{35}S]-methionine (1 μCi ml^{-1}). After 1 min, half of the cells were removed and centrifuged at 21,000 x g for 5 min. The cell paste was washed once in TEMMB buffer and resuspended in TEMMB buffer. The cell suspension was immediately frozen in liquid nitrogen and stored at -20°C until required.

The incorporation of [^{35}S]-methionine into the remaining cells, after the 1 min sample, was stopped by diluting out with cold methionine (50 mM final concentration). These were harvested as described above after a further 5 min incubation. Soluble protein extracts were prepared from the labelled cells and subjected to SDS-polyacrylamide gel analysis, using the purified RuBisCo from R. blastica as marker proteins. The gels were stained with coomassie blue to locate the polypeptide bands. After destaining, the gels were subjected to fluorography to locate the

radioactively labelled polypeptides.

2.25 Fluorographic Detection of Radioactivity in Polyacrylamide Gels

The fluorographic technique developed by Bonner and Laskey (1974) and Laskey and Mills (1975) was used for the detection of radioactively labelled proteins in polyacrylamide gels. In this procedure, SDS-polyacrylamide gels were run in the usual manner and the gels fixed in a solution of 15% (v/v) TCA and 45% (v/v) methanol for at least 1 h. After fixation, the gel was soaked in 500 ml of DMSO for ½ h and the solution changed for another ½ h. The gel was then put into 200 ml of 20% (w/v) solution of PPO in DMSO for 3 h after which it was washed in 500 ml of dionized water with several changes. After washing, the swollen gel was soaked in 50% (v/v) methanol (to shrink the gel) and it was subsequently dried down onto a 3 M Whatman paper. The dried gel was placed in contact with X-ray film (Kodak X-Omat S), and kept at -80°C during exposure for about 3 to 5 days. The exposed film was subsequently developed for 3 min using Kodak LX24 X-ray developer and then fixed using Kodafix FX40. The developed film was washed for several minutes in running tap water and finally rinsed in distilled water. After washing, the film was air-dried at room temperature.

Chapter 3

Results and Discussion I

Ribulose Bisphosphate Carboxylase/Oxygenase and CO_2 Fixation in the Rhodospirillaceae

3.1 Introduction

The species of the purple non-sulphur photosynthetic bacteria (Rhodospirillaceae) which were orginally classified into four genera primarily on the cell morphology (Pfennig and Trupper, 1971, 1983), have recently been rearranged into six genera on the basis of morphological and physiological properties (Imhoff et al., 1984). The Rhodopseudomonas species (Pfennig, 1977) present a large spectrum of overlapping morphological, biochemical and physiological properties. However, on the basis of the cell cycle complexity, it is possible to arrange the organisms of the Rhodospirillaceae into a morphogenetic gradient (Figure 3.1) which at least may be indicative of morphogenetic evolution (Whittenbury and Dow, 1977; Kelly and Dow, 1984). Within these groups of bacteria three main cell cycle types are easily recognised: 1) the simple monomorphic type as in Rhodospirillum rubrum. 2) the dimorphic cell cycle type exhibited mainly by the Rhodopseudomonas and 3) the complex polymorphic cell cycle of Rhodomicrobium. e.g. R. vannielii (Dow, 1974).

R. rubrum reproduces by binary fission, giving rise to cells in which the "mother and daughter" cells are morphologically and physiologically the same. The species of bacteria of the genus Rhodopseudomonas are divided into three main groups (Pfennig, 1977). The cell cycle of the organisms in Group I to which R. sphaeroides and R. capsulata belong are less well understood. While it has been suggested that these organims reproduce by binary fission, (Imhoff et al., 1984) it is believed that R. sphaeroides strain cordata may have a more complex life cycle (Gest et al., 1983). However, R. sphaeroides and R. capsulata have some ultrastructural properties (e.g. vesicular type of photosynthetic membrane and large type cytochrome C_2) similar to R. rubrum. In the group III to which

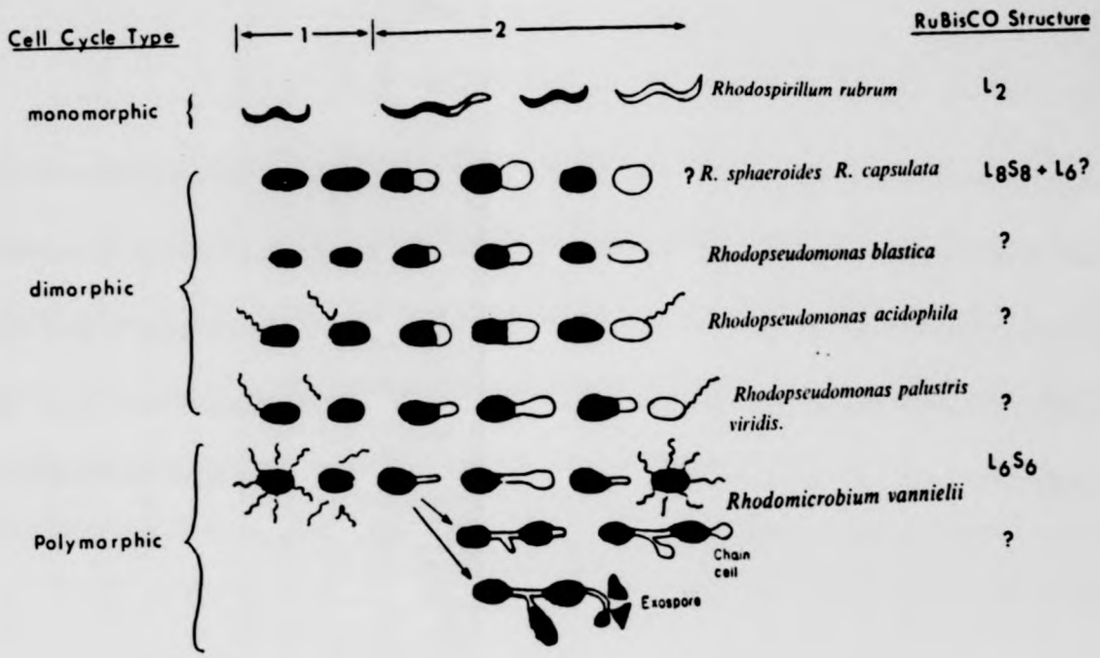


Figure 3.1 A morphogenetic gradient of the cell cycles of several phototrophic purple nonsulphur bacteria

1) the maturation phase; 2) the reproductive phase

(Modified from Kelly and Dow, 1984).

R. palustris, R. viridis and R. acidophila belong, dimorphism is clearly evident. These organisms reproduce by binary fission producing progeny in which the "mother and daughter" cells are morphologically and physiologically different. R. blastica on the other hand, divides symmetrically, and hence produces progeny which are apparently identical morphologically but which are thought to be functionally distinct cell types (Eckersley and Dow, 1980). However, this organism has a number of properties which are exhibited by other members of the Rhodospirillaceae. For instance, its morphology, mode of growth and its photosynthetic membrane system resembles those of the group III, while its cell cycle simplicity, photopigment compositions and physiology are more related to that of the group I Rhodopseudomonas (Eckersley and Dow, 1980). In fact, the morphological and physiological properties exhibited by R. blastica has lead Imhoff and co-workers (Imhoff et al., 1984) to suggest that this organism should be placed in another branch of "super family IV".

Rhodomicrobium vannielii (Rm5) has a complex polymorphic cell cycle type (Figure 3.1). This organism can form three distinct cell types: swarmer cells, multicellular arrays, i.e. chain cells and exospores depending on the cultural conditions (Whittenbury and Dow, 1977; Dow et al., 1985). The mode of cell differentiation and its regulation has been reviewed elsewhere (Whittenbury and Dow, 1977; Dow et al., 1983). It is however, evident from this short survey of cell cycle complexity that the vast majority of species within the Rhodospirillaceae express different morphologial and physiological cell types during growth. The biochemical and physiological data thus far reported using the batch cultures of these organisms may represent an average for a heterogeneous population and may not be valid for individual cells in the population. Attention

is therefore drawn to the development of techniques for obtaining homogeneous, i.e. synchronous cultures for biochemical analysis.

The limited information that is available regarding RuBisCO and CO_2 fixation in the Rhodospirillaceae have been obtained from studies with R. rubrum. which is not only morphologically very simple, but also possesses the smallest, and probably the most primitive form of RuBisCO thus far characterised (Tabita and McFadden, 1974a, b). This organism synthesises a dimer of large subunits of RuBisCO. However, it is currently being suggested that a small subunit may be burried within the carboxyl terminal of the enzyme (T. J. Andrews, personal communication). Evidence in support of this view comes from the fact that the DNA sequence of the carboxyl terminal of RuBisCO enzyme from R. rubrum bears some sequence homology to the small subunit of the spinach enzyme (T. J. Andrews, personal communication). Further evidence to rigorously support this view is awaited.

Little is known about RuBisCO and CO_2 fixation in the Rhodopseudomonas. Although it has been shown that R. sphaeroides and R. capsulata have two forms of RuBisCO (Gibson and Tabita, 1977a, b), the regulation of synthesis of these enzymes and the physiological significance of their presence is unknown. In the other groups of Rhodopseudomonas , where dimorphism is a prominent feature, it is not known how CO_2 fixation and the synthesis of the key enzymes of the Calvin cycle, e.g. RuBisCO is regulated in the different cell types (i.e. the "mother and daughter" cells). It is also not known whether the dual enzyme system (2 forms of RuBisCO), as expressed in R. sphaeroides and R. capsulata. is a consequence of dimorphism, i.e. do the different cell types synthesise different molecular forms of the RuBisCO enzyme?

The CO_2 fixation and RuBisCO in R. vannielii - an organism which is morphologically at least the most complex of the Rhodospirillaceae (Figure 3.1) has been examined (Taylor, 1979). Evidence suggests that the RuBisCO from R. vannielii is evolutionarily more advanced than the enzyme from R. rubrum in that it has properties closely related to the plant enzymes although it is thought to have an L_6S_6 configuration (Taylor, 1979; Taylor and Dow, 1980). However, Taylor (1979) used R. vannielii with the "simplified cell cycle" type in which the chain cells and exospores are not expressed. It is therefore not known whether a heterogeneous culture of R. vannielii synthesises different molecular forms of RuBisCO or not.

In order to find answers to some of the questions raised above, it is desirable to develop methodology which can be used for rapid screening of the different molecular forms of RuBisCO and also for studying the physiology of the sythesis of this enzye from photosynthetic prokaryotes. In this respect a rapid method (using sucrose gradient centrifugation) was developed for the isolation of the RuBisCO enzyme(s) from a number of bacterial sources. In addition, an in situ non-denaturing polyacrylamide gel assay for RuBisCO was developed for the quick screening for the different molecular forms of RuBisCO folloiwng sucrose gradient centrifugation. Most of the work in this study was done using Rhodopseudomonas blastica because of its morphological and physiological advantage over other members of the Rhodospirillaceae: the organism is morphologically simple, reproduces by binary fission giving rise to cells which are morphologically similar but physiologically distinct. It has some morphological and physiological properties exhibited by various species of the Rhodospirillaceae. In addition, it grows aerobically both in the light and in the dark, and anaerobically in the light.

R. blastica appears to be a 'transition' organism (in terms of morphogenetic evolution, Figure 3.1) between the less advanced (e.g. R. sphaeroides) and more advanced (e.g. R. palustris. R. vannielii) of the Rhodospirillaceae.

3.2 The Growth Physiology of Rhodopseudomonas blastica

As with other members of the Rhodospirillaceae. R. blastica utilizes a wide range of organic compounds (with the exception of alcohols) to support growth (Eckersley and Dow, 1980). In addition, it utilizes monosaccharides and grows photolithotrophically on H_2 and CO_2. Although for photosynthesis, R. blastica requires anaerobic conditions, the organism also grows both in the dark and in the light under aerobic conditions.

Since the bulk of the work in this thesis was done with R. blastica. it is necessary to investigate its growth physiology on different carbon substrates. In this respect, R. blastica was grown on mineral salts medium supplemented with different carbon sources, and the growth was followed by measuring the culture absorbance at 650 nm with time. Figure 3.2 shows the growth curves of R. blastica on different carbon substrates under anaerobic conditions at the light intensity of 2000 lux. Three distinct phases of growth, i.e. the lag, the exponential and stationary, were evident when the various carbon sources were tested either individually or as mixtures. With the exception of growth on butyrate and on mineral salts medium, the lag phase lasted for about 12 h. A 36 h lag phase was observed for growth on butyrate medium which lacked bicarbonate, while on mineral salts medium alone, growth was

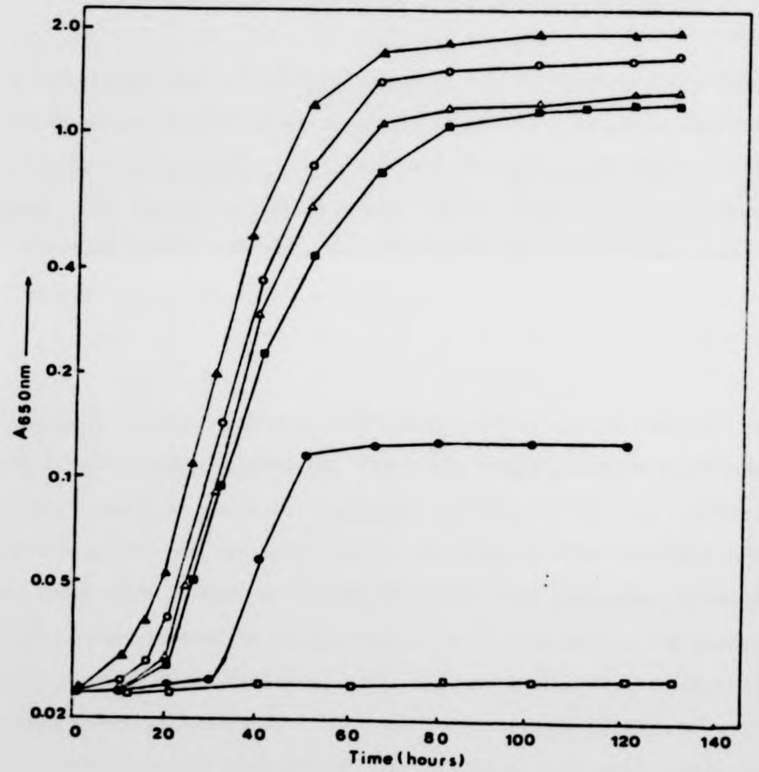


Figure 3.2 Growth of Rhodopseudomonas blastica on different carbon substrates

R. blastica was grown in batch culture under anaerobic conditions as described in materials and method (Section 2.8.1) Growth was followed by measuring the culture absorbance at 650nm. The mineral salts medium contained the following carbon substrates:

Pyruvate + malate	(▲)
Butyrate + bicarbonate	(O)
Malate	(△)
Pyruvate	(■)
Butyrate	(●)
Mineral salts medium	(□)

negligible. In all cases, stationary phase of growth was reached after 72 h. The growth rate (0.14 h^{-1}) was not affected by the different carbon substrates. However, the cell densities were highest (550 to 600 mg dry wt. l^{-1} culture) when the organism was grown on either pyruvate-malate or butyrate-bicarbonate medium. The inclusion of bicarbonate into pyruvate and the malate media did not affect the growth rate and the cell density. When R. blastica was grown on butyrate medium which lacked bicarbonate, growth was poor with the result that cell density did not exceed an absorbance of 0.14 (45 mg dry wt l^{-1}).

The lack of growth of R. blastica on mineral salts medium is suggestive of absolute requirement for carbon source for the synthesis of the cell materials. The addition of CO_2, furnished as bicarbonate to the culture is only essential in those instances where CO_2 cannot be generated from the organic electron donor, e.g. butyrate (Doelle, 1975), presumably because it is more reduced than the intracellular milieu (Tabita, 1981). As will be shown later, malate and pyruvate yield considerable amounts of CO_2 during growth, and consequently the addition of bicarbonate to the growth medium is unnecessary. On the basis of growth rate and cell density achieved in this study, pyruvate-malate or butyrate-bicarbonate medium (unless otherwise stated) were chosen as the growth medium for either small or large culture of R. blastica.

3.3 CO_2 Fixation by Rhodopseudomonas blastica

The requirements for CO_2 fixation by whole cells of R. blastica was investigated with cells grown photoheterotrophically on defined media. Cells were harvested from exponential phase of growth, resuspended in the

various growth media, and the incorporation of $NaH^{14}CO_3$ into the cells was followed over a period of 45 min. A linear rate of $^{14}CO_2$ fixation was observed over a period of 2 to 25 min when cells of R. blastica were resuspended in complete (defined) growth media and incubated in the light (Figure 3.3). The rate of $NaH^{14}CO_3$ incorporation was highest in cells resuspended in butyrate-HCO_3 medium. The in vivo rate of incorporation of $NaH^{14}CO_3$ into whole cells grown on pyruvate-malate was lower, presumably because significant proportion of the organic carbon is derived from either pyruvate or malate in the medium. In the absence of light and consequently energy supply (i.e. dark anaerobic incubations), $^{14}CO_2$ incorporation was negligible, although under this condition, cells were still viable over the incubation period.

Furthermore, cells resuspended in basal salts showed a lower rate of $^{14}CO_2$ incorporation than cells resuspended in the pyruvate-malate medium. This might indicate the requirement for reductant for $^{14}CO_2$ fixation.

The decrease in the rate of $NaH^{14}CO_3$ incorporation after 25 min of incubation was observed for all growth media tested (Figure 3.3). This may be due to the dissociation of $[^{14}C]\text{-}HCO_3^-$ and the equilibrium of CO_2 between its various forms as shown by the equation:

$$CO_2 + H_2O \underset{}{\overset{(1)}{\rightleftharpoons}} H^+ + HCO_3^- \overset{(2)}{\rightleftharpoons} H^+ + CO_3^{2-}$$

The dissociation constants (K diss at 30°C) for reactions (1) and (2) are 5.7×10^{7} and 4.8×10^{-11} respectively (Weast, 1975). Christeller and Laing (1978) showed that HCO_3^- is the active species taken up by cultures of Rhodospirillum rubrum but CO_2 is the active species fixed therefore, the addition of labelled HCO_3^- to the resuspended cells of R. blastica

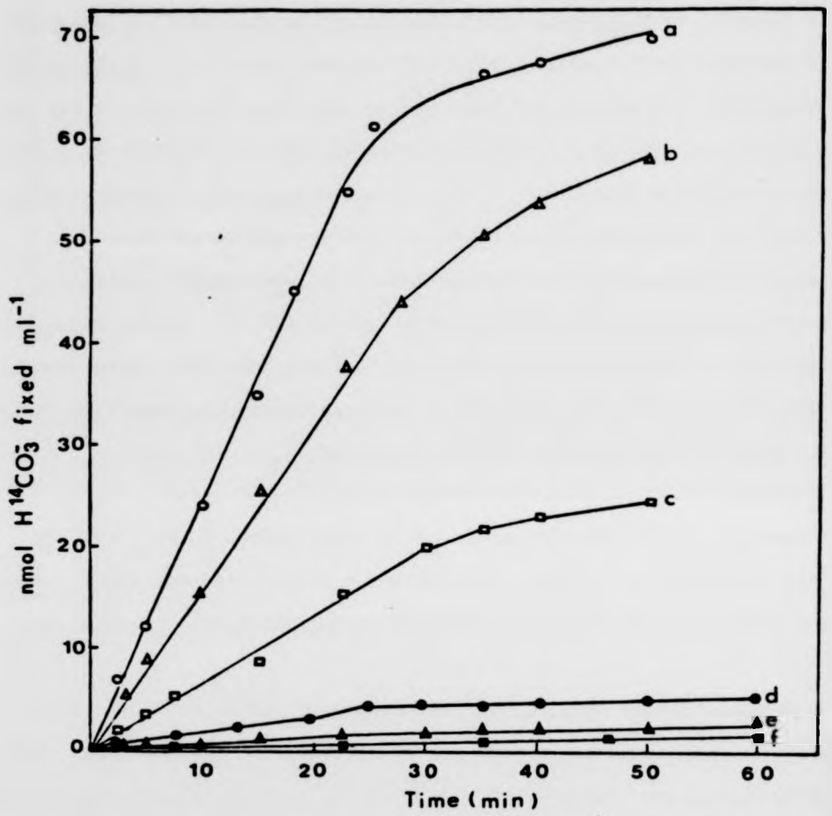


Figure 3.3 $^{14}CO_2$ fixation by intact cells of Rhodopseudomonas blastica

a) Butyrate - bicarbonate + light

b) Pyruvate + malate + light

c) Mineral salts medium + light

d) Butyrate - bicarbonate + dark

e) Pyruvate - malate + dark

f) Killed cells

would give an initial high specific activity, since the predominant species at early fixation will be $H^{14}CO_3^-$ which is the form most likely taken up by the cells. As the equilibrium is reached between CO_2, HCO_3^- and CO_3^{2-} species, the effective concentration of HCO_3^- is decreased. This may account for the decrease in the rate of $^{14}CO_2$ fixation after the 25 min incubation.

The calculated specific activity of 4.1 umol of $^{14}CO_2$ fixed.min^{-1} (g dry wt of cells)$^{-1}$ on butyrate-bicarbonate medium, is comparable to that reported for heterotrophic growth of R. rubrum on similar medium (Sarles and Tabita, 1983). However, as will be shown later, the rate ofCO_2 fixation by intact cells of R. blastica is growth conditions dependent. In conclusion, the results discussed herein, show that both light and reductant are required for CO_2 fixation by intact cells of R. blastica. This is an indication of the energy dependent CO_2 fixation in this organism.

3.4 The Key Enzymes of the Calvin Cycle in Rhodopseudomonas blastica

The presence of the key enzymes of the Calvin cycle in R. blastica was investigated by using ribulose bisphosphate (RuBP) and ribulose monophosphate (RuMP) as the potential substrates for CO_2 fixation by the soluble protein extracts of this organism. The effect of NADH as a reductant, and ATP and AMP additions, on the rate of CO_2 fixation was also examined. The soluble protein extracts were prepared from cells grown photoheterotrophically on butyrate-bicarbonate medium.

Table 3.1 shows the effect of the different substrates on the rate of CO_2

Table 3.1 Effect of substrates on CO_2 fixation by the soluble protein extracts of Rhodopseudomonas blastica grown photoheterotrophically on butyrate-bicarbonate medium

Substrate+	Enzyme Activity (nmol of $^{14}CO_2$ fixed.min^{-1}.mg protein^{-1})
None	0.0
ATP	0.02
NADH	0.01
NADH + ATP	0.05
AMP	0.0
RuBP	73.5
RuBP + NADH	76.0
RuBP + ATP	74.0
RuBP + NADH + ATP	78.2
RuMP	1.3
RuMP + ATP	39.4
RuMP + NADH	24.2
RuMP + ATP + NADH	68.2
RuMP + AMP	0.5

+ ATP, AMP, and NADH were added to a final concentration of 0.2 µmol

fixation by the soluble protein extracts of R. blastica. In the absence of any substrate, or the addition of ATP, AMP and NADH either individually or in combination, CO_2 fixation rate was negligible (ranging from 0-0.05 nmol min^{-1}.mg $protein^{-1}$) (Table 3.1). When RuBP was added (0.75 mM final concentration) as the substrate, the rate of CO_2 fixation increased from 0 to 76 nmol.min^{-1}.mg $protein^{-1}$. The RuBP dependent CO_2 fixation by the soluble protein extracts of R. blastica indicate the presence of ribulose bisphosphate carboxylase in this organism. With RuBP, an absolute requirement for ATP and $NADH_2$ for activity was not observed. On the other hand, when RuMP was used as the test substrate, ATP and NADH were required for maximal activity. The ATP requirement is in agreement with RuMP requiring conversion to RuBP by phosphoribulokinase before CO_2 fixation can occur. The phosphoribulokinase activity from a number of photosynthetic bacteria (Tabita, 1981; Bowien, 1983), and a majority of the hydrogen bacteria (MacElroy et al., 1969; Siebert et al., 1981) has been reported to be enhanced by NADH. Although NADH was found to enhance the activity of phosphoribulokinase activity in the soluble extract of R. blastica. unlike R. sphaeroides and Nitrobacter winograsdskyi (Rindt and Ohman, 1969; Kiewsow et al., 1977), an absolute requirement was not observed, since there was an appreciable kinase activity in the absence of NADH (Table 3.1). However, this may be due to the intracellular NADH present in the soluble protein extracts. The influence of NADH on the activity of phosphoribulokinase from this organism requires studies on the purified enzyme . The phosphoribulokinase activity in the soluble extract of R. blastica was inhibited by AMP, as evident from its lower activity when AMP was used together with RuMP.

The presence of RuBP carboxylase and phosphoribulokinase in the soluble

extracts of R. blastica shows that the Calvin cycle of CO_2 fixation operates in this organism. This observation will be in accordance with the energy dependent CO_2 fixation by whole cells of R. blastica as demonstrated before (section 3.3). Furthermore, the stimulation of CO_2 fixation by ATP and NADH in the presence of RuMP and its inhibition by AMP indicate that phosphoribulokinase may be a site of the in vivo regulation of CO_2 fixation in this organism. This may argue for a regulation of the Calvin cycle enzymes according to the energetic state of the cells. This control pattern is physiologically reasonable since synthesis of cell carbon from CO_2 requires an ample supply of reducing power and energy.

3.5 Summary of Observations on the Physiology of Rhodopseudomonas blastica

In this study it is shown that Rhodopseudomonas blastica utilizes a number of oxidizable organic compounds for growth. Growth was best either on pyruvate, malate or a combination of both. Growth on reduced organic compounds such as butyrate require the presence of exogenous CO_2 which can be supplied as sodium bicarbonate in the growth medium. In the absence of carbon substrates there was no growth.

It is shown in this study that whole cells of R. blastica require light and reductant for $^{14}CO_2$ fixation, indicating energy dependent CO_2 fixation by this organism.

Evidence also shows that Rhodopseudomonas blastica possess the key enzymes of the Calvin cycle namely, ribulose bisphosphate carboxylase and phosphoribulokinase, and this suggests that the ribulose bisphosphate pathway (Calvin cycle) is the main path of CO_2 fixation in this organism. Furthermore, $^{14}CO_2$ by the soluble extract of R. blastica was stimulated by ATP and NADH in the presence of ribulose monophosphate (RuMP) but inhibited by AMP. This observation suggests that phosphoribulokinase may be a site of in vivo regulation of CO_2 fixation in this organism.

3.6 Purification of RuBisCO from the Rhodospirillaceae by sucrose gradient centrifugation

3.6.1 Introduction

The use of sucrose density velocity centrifugation for the purification of ribulose bisphosphate carboxylase/oxygenase (RuBisCO) from leaf extract was first reported by Goldwaite and Bogorad (1971). This technique relied on the high sedimentation coefficient and molecular weight of the plant RuBisCO compared with the bulk of the soluble proteins. In plants where RuBisCO may constitute more than 60% (w/w) of the total soluble protein (Ellis, 1979), the one step sucrose gradient purification procedure may yield homogeneous enzyme (Goldwaite and Bogorad, 1971). Tabita and McFadden (1974c, 1976) used sucrose gradient velocity centrifugation to partially purify RuBisCO from a number of prokaryotic sources. In this technique, swing-out rotors were used and this consequently involved long hours (up to 24 h) of centrifugation. Flamm et al. (1966) first showed the advantages of using fixed angle as opposed to swing-out rotors for the equilibrium sucrose gradient centrifugation in the separation of nucleic acid and the mixtures

thereof. The technique was extended by Covey and Taylor (1980) for the purification of RuBisCO from Rhodomicrobium vanielii. Covey and Taylor (1980) showed a 50-fold enrichment for RuBisCO when soluble extracts of R. vannielii was centrifuged into linear sucrose gradient (0.2-0.8 M sucrose) in a fixed angle rotor at 240,000 x g for 1. h. However, such enzyme preparations still showed a number of contaminants when analysed on SDS-polyacrylamide gels.

There are some reports in literature concerning the unstable nature of RuBisCO from a number of prokaryotic sources. For instance, Taylor (1979) showed that purified RuBisCO from R. vannielii was unstable, losing 50% of its activity after storage at 2°C for 21 days. Furthermore, Taylor and Dow (1980) showed that the purified RuBisCO from R. vannielii lost its small subunit after ammonium sulphate precipitation and the subsequent storage at 4°C for 14 days. Similarly, Codd and Stewart (1977) and Andrews and Ballment (1983) showed the relative ease by which the small subunit of RuBisCO may be lost by acid precipitation treatment. These observations may have accounted for the inconsistences in the quaternary structures of some purified RuBisCO from a number of prokaryotic sources (see Section 1.5.3.2 of this thesis).

In view of the unstable nature of some prokaryotic RuBisCO and the possibility of loss of subunits during lengthy purification procedures, a single step purification procedure was developed to isolate RuBisCO from a number of bacteria of the Rhodospirillaceae. This method involves the use of the step sucrose gradient centrifugation in a fixed angle rotor for 2.5h. An in situ assay for RuBP carboxylase in polyacrylamide gels was also developed to screen for the presence of one or more different molecular forms of the enzyme in the soluble extracts of a number of

photosynthetic bacteria.

3.6.2 Comparisons of the step and linear sucrose gradient centrifugation for the isolation of ribulose bisphosphate carboxylase/oxygenase from Pseudomonas oxalaticus

Step and linear sucrose gradient centrifugation were used for partial purification of ribulose bisphosphate carboxylase/oxygenase from P. oxalaticus. The results are then compared, with a view to determine which of the two methods gives a better resolution. Soluble protein extracts of P. oxalaticus grown autotrophically on formate was centrifuged into the gradients as described under Materials and Methods. After centrifugation, the gradients were harvested and the RuBP dependent $^{14}CO_2$ fixation of the 1 ml fractions was determined. The distribution of protein within the fractions was then examined by SDS-polyacrylamide gel electrophoresis. Figure 3.4 shows the 10% (w/v) polyacrylamide slab gels of the SDS-dissociated proteins from the fractions of the sucrose gradient centrifugation. In either case, the bulk of the soluble protein remained at the top of the gradients, as evident from the Cooomassie stained polypeptides in the gels (Figure 3.4, lanes 10 to 13). Ribulose bisphosphate carboxylase/oxygenase from the soluble extract of P. oxalaticus was however, concentrated into three fractions (representing about 1/5th of the total gradient size) after centrifugation into 0.2 to 0.8 M step sucrose gradient. This is evident from the presumably stained large subunits of the enzyme (Figure 3.4a, lanes 7, 8 and 9). On the other hand, when the soluble protein extracts of P. oxalaticus was centrifuged into linear sucrose gradient, ribulose bisphosphate carboxylase/oxygenase was spread across 1/2of the gradient size(figure 3.4b, lanes 6 to 12). The linear sucrose gradient centrifugation is

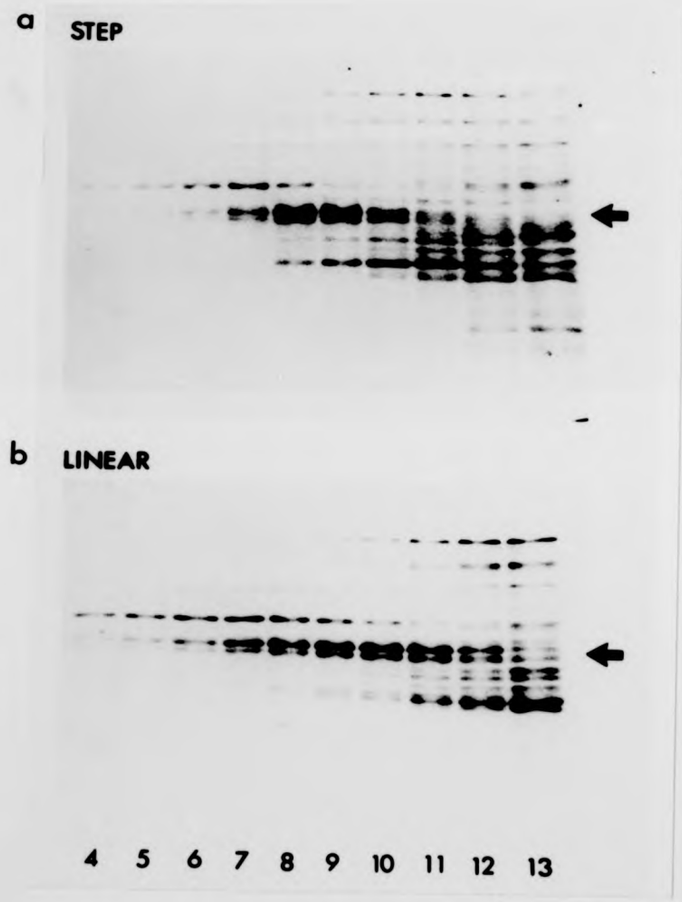


Figure 3.4 10% (w/v) SDS Polyacrylamide gel electrophoresis of fractions from 0.2-0.8 M sucrose gradient centrifugation of the soluble protein extracts of Pseudomonas oxalaticus

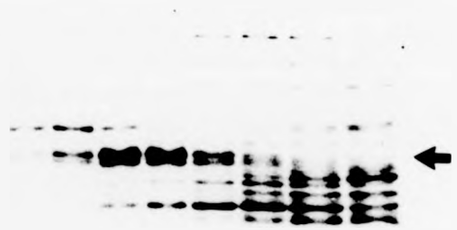
The large subunit (L) of the ribulose bisphosphate carboxylase is arrowed.

The numbers represent fractions from the bottom of the gradients.

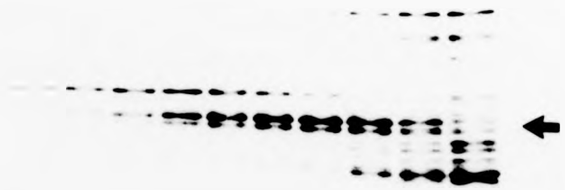
Coomassie blue stain.

Figure 3.4 10% (w/v) SDS Polyacrylamide gel electrophoresis of fractions from 0.2-0.8 M sucrose gradient centrifugation of the soluble protein extracts of Pseudomonas oxalaticus

a STEP



b LINEAR



4 5 6 7 8 9 10 11 12 13

The large subunit (L) of the ribulose bisphosphate carboxylase is arrowed.

The numbers represent fractions from the bottom of the gradients.

Coomassie blue stain.

considered therefore, not to be a suitable method for the isolation of the intermediate size class (Mr = 360,000, Lawlis et al., 1979) of RuBisCO from P. oxalaticus. Goldwaite and Bogorad (1971) reported that RuBisCO from the soluble protein extracts of spinach leaves could be purified to virtual homogeneity by centrifugation on a linear sucrose gradient for 20 h using a swing-out rotor. The differences in the resolution reported by the authors and that discussed herein, may be a consequence of different centrifugation procedures and the fact that the enzyme from the spinach leaves is of the large molecular weight class (Mr = 550,000, Kawashima and Widman, 1970).

Undescribed preliminary experiments show that the sedimentation pattern of RuBisCO on the step sucrose gradient is affected by centrifugation periods, e.g. when gradients were centrifuged for up to 3.5h at 240,000 x g, the enzyme was pelleted to the bottom of the centrifuge tube, thereby leading to contamination by higher molecular weight substances in the soluble extracts. This efect may however, be eliminated by using higher volumes of the 0.8 M sucrose as a cushion, though this required a reduction in the volume of the sample that can be applied to the gradient.

In conclusion, the single step purification procedure developed for the isolation of ribulose bisphosphate carboxylase/oxygenase from P. oxalaticus is rapid, mild and requires little sample (maximum of 2 ml) to be analysed. Step sucrose gradients were found to be more satisfactory (i.e. better resolution) than the linear sucrose gradients in this purification procedure.

The one step purification procedure using step sucrose gradient may prove

useful for isolating RuBisCO from a wide range of photosynthetic prokaryotes where various classes of this enzyme are a prominent feature (McFadden, 1980).

3.6.3 Partial Purification of Ribulose bisphosphate carboxylase/oxygenase from the Rhodospirillaceae

The one step sucrose gradient centrifugation method developed for the partial purification of ribulose bisphosphate carboxylase/oxygenase from P. oxalaticus was used to isolate the enzyme from a number of photosynthetic bacteria of the Rhodospirillaceae. The enzyme preparation from sucrose gradient centrifugation was analysed on non-denaturing tube gels to ascertain its purity. The gels were then subjected to in situ assay for RuBisCO with a view to screening for the presence of one or more different molecular forms of this enzyme in the organisms investigated.

a) Rhodopseudomonas blastica

The sucrose gradient centrifugation procedure was used to isolate RuBisCO from R. blastica. Figure 3.5 shows A_{280} nm of soluble protein extract from photoheterotrophically grown cells of R. blastica (on pyruvate-malate medium) centrifuged into a 0.2-0.8 M step sucrose gradient. The RuBP dependent CO_2 fixation of the 1 ml fractions is superimposed. A broad peak of CO_2 fixing activity separated from the bulk of the soluble protein was obtained (Figure 3.5a). This might indicate the presence of more than one molecular form of RuBisCO in the soluble extract of R. blastica. To investigate this prediction, active RuBisCO fractions from the 0.2-0.8 M sucrose gradient centrifugation were pooled and electrophoresed on 5% (w/v) polyacrylamide non-denaturing tube gels. Duplicate gels were run, one which was stained with Coomassie blue to

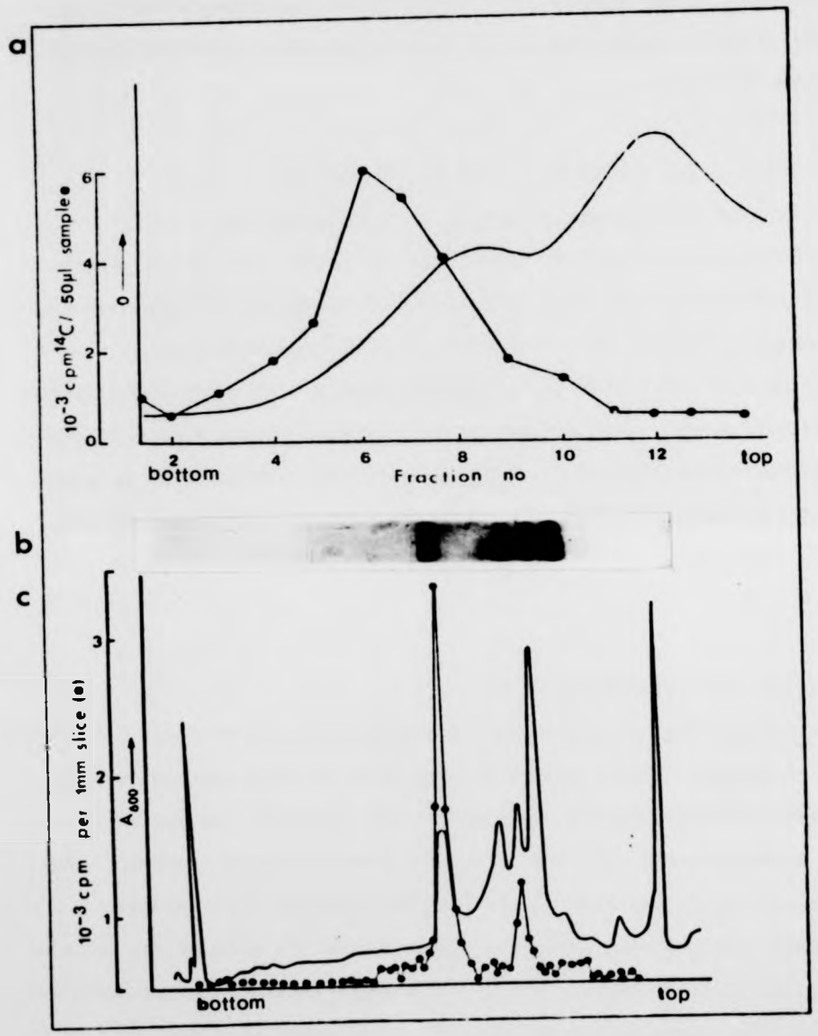
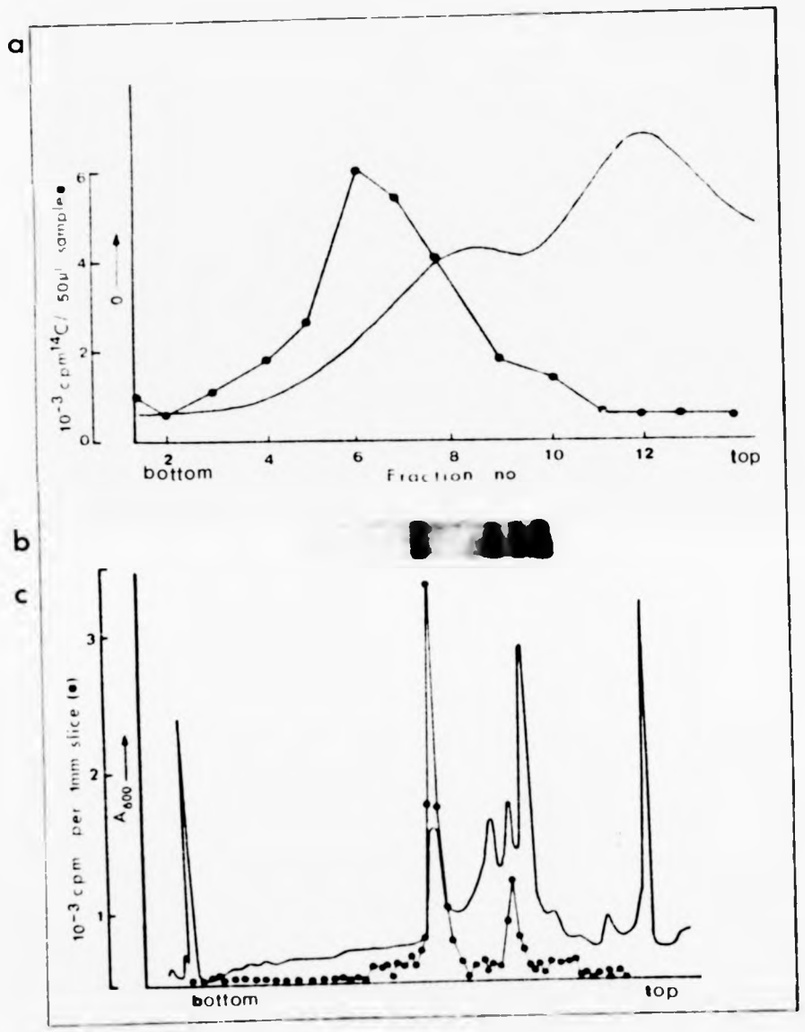


Figure 3.5 Isolation of ribulose bisphosphate carboxylase/oxygenase from Rhodopseudomonas blastica by sucrose gradient centrifugation

a) A_{280} nm of the UV absorbing material and RuBP dependent $^{14}CO_2$ fixing activity of the 1 ml fractions of the soluble extract of R. blastica centrifuged into 0.2 to 0.8 M step sucrose gradient. The gradient was harvested from the bottom.

b) Non-denaturing polyacrylamide gel electrophoresis of the pooled active RuBisCO fractions from sucrose gradients shown in (a). 40 ug of protein was applied to the gel polymerised from 5% (w/v) acrylamide.

c) Densitometric scan of the stained gel upon which the RuBP dependent $^{14}CO_2$ fixing activity of the 1 mm slices is superimposed.



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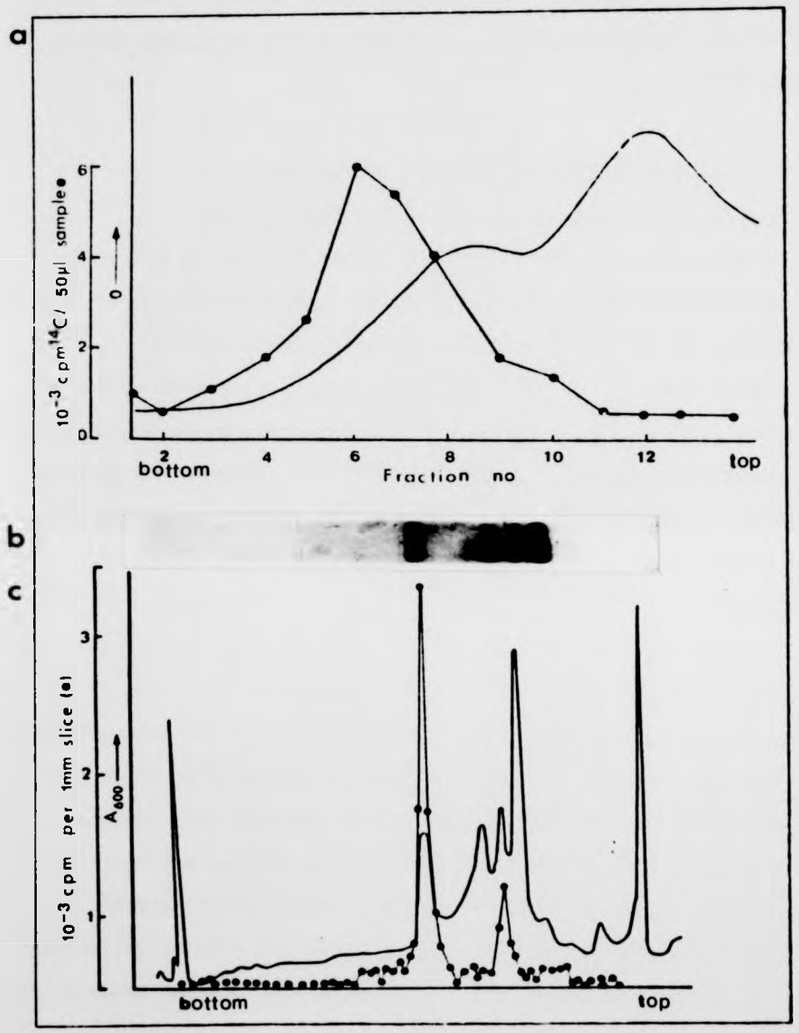

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c) Densitometric scan of the stained gel upon which the RuBP dependent $^{14}CO_2$ fixing activity of the 1 mm slices is superimposed.

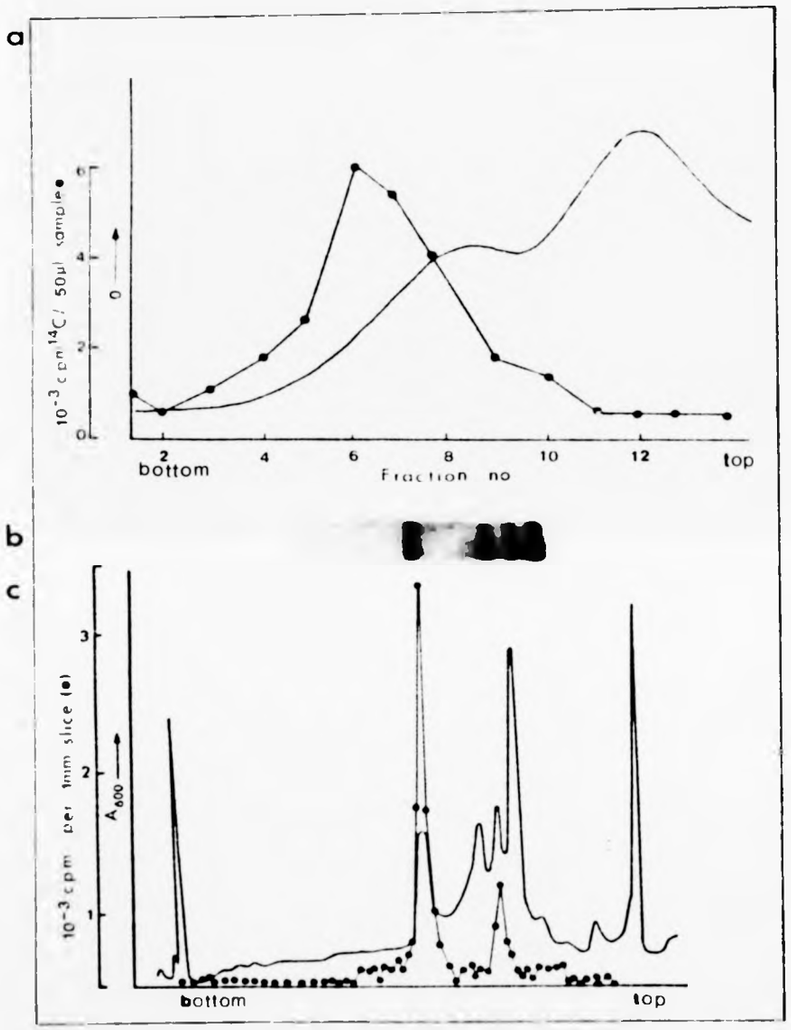

Figure 3.5 Isolation of ribulose bisphosphate carboxylase/oxygenase from Rhodopseudomonas blastica by sucrose gradient centrifugation

a) A_{280} nm of the UV absorbing material and RuBP dependent $^{14}CO_2$ fixing activity of the 1 ml fractions of the soluble extract of R. blastica centrifuged into 0.2 to 0.8 M step sucrose gradient. The gradient was harvested from the bottom.

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c) Densitometric scan of the stained gel upon which the RuBP dependent $^{14}CO_2$ fixing activity of the 1 mm slices is superimposed.

locate protein bands in the gel, after which it was scanned at 600 nm. Four well resolved bands were obtained after staining and destaining (Figure 3.5b). To locate the enzymic activity in the gel, one of the duplicate gels was sliced into 1 mm and each assayed for RuBP dependent-CO_2 fixing activity. Two distinctive peaks of enzymic activity were obtained from the gel slices (Figure 3.5c). The two peaks were separated by about 20 mm. A trace of the densitometric scan (A_{600}) of the stained gel is shown in Figure 3.5c. The relative levels of the two forms of the RuBisCO enzyme in the stained gel was estimated from the weight of the area which correspond to each stained band on the densitometric trace. The results show that the low molecular weight (faster migrating) and the higher molecular weight (slower migrating) forms of the RuBisCO from photoheterotrophically (on pyruvate-malate) grown cells were present in the 3:1 ratio. Similarly, the RuBP dependent $^{14}CO_2$ fixing activity of the low molecular form in the gel piece was about three times higher than the higher molecular weight enzyme. Furthermore, the two peaks of enzyme activity were always present in the relative level stated above, whether or not phenylmethylsulfonyl fluoride (PMSF), a protease inhibitor, was included in the growth medium or in the soluble extract preparations before the sucrose gradient centrifugation and the subsequent gel analysis. This suggests that the two forms of the RuBisCO enzyme are not degradative products resulting from protease activity. However, the enzyme fractions were contaminated by other proteins as shown in non-denaturing gels (Figure 3.5b). Other methods were therefore, sought to purify the enzymes from R. blastica. The results of the investigations are discussed in Section 4 of this thesis.

b) Rhodopseudomonas sphaeroides

The possibility of using the one step sucrose gradient centrifugation

method to isolate the two different molecular forms of RuBisCO reported in Rhodopseudomonas sphaeroides (Gibson and Tabita, 1977a) was investigated. The soluble extract of R. sphaeroides at late exponential phase of growth on butyrate bicarbonate medium was used. Figure 3.6a shows A_{280} nm of soluble extract of R. sphaeroides centrifuged into 0.2-0.8 M step sucrose gradient on which the RuBP dependent $^{14}CO_2$ fixing activity of the 1 ml fractions is superimposed. A broad peak of enzyme activity was separated from the bulk of of the soluble protein which remained at the top of the gradient. Active fractions (5, 6, 7 and 8) were pooled and electrophoresed on 5% (w/v) non-denaturing tube gels. The gels were then analyzed by the in situ polyacrylamide gel assay for ribulose bisphosphate carboxylase as described before. Figure 3.6b shows the electrophoretogram of the stained gel along with the corresponding activity profile in the gel slices. Two major peaks of activity are associated with two stained protein bands on the gel. The higher molecular weight (slower migrating from the top of the gel) form of the RuBisCO enzyme had activity which was about the same as the lower molecular weight (faster migrating from the top of the gel) (Figure 3.6b). Furthermore, a densitometric scan of the stained gel at 600 nm show that the high molecular weight form were present in the 1:1 ratio. However, the protein band corresponding to the low molecular weight form of the RuBisCO enzyme was more diffused than the band corresponding to the high molecular weight form. The two different molecular forms of ribulose bisphosphate carboxylase/oxygenase isolated from the soluble extract of Rhodopseudomonase sphaeroides by one step sucrose gradient centrifugation probably represent the Form I and the Form II RuBisCO reported in this organism by Gibson and Tabita (1977a). The properties of the homogeneous enzymes have been discussed by Gibson and Tabita (1977), therefore no further attempt was made in this study to purify the

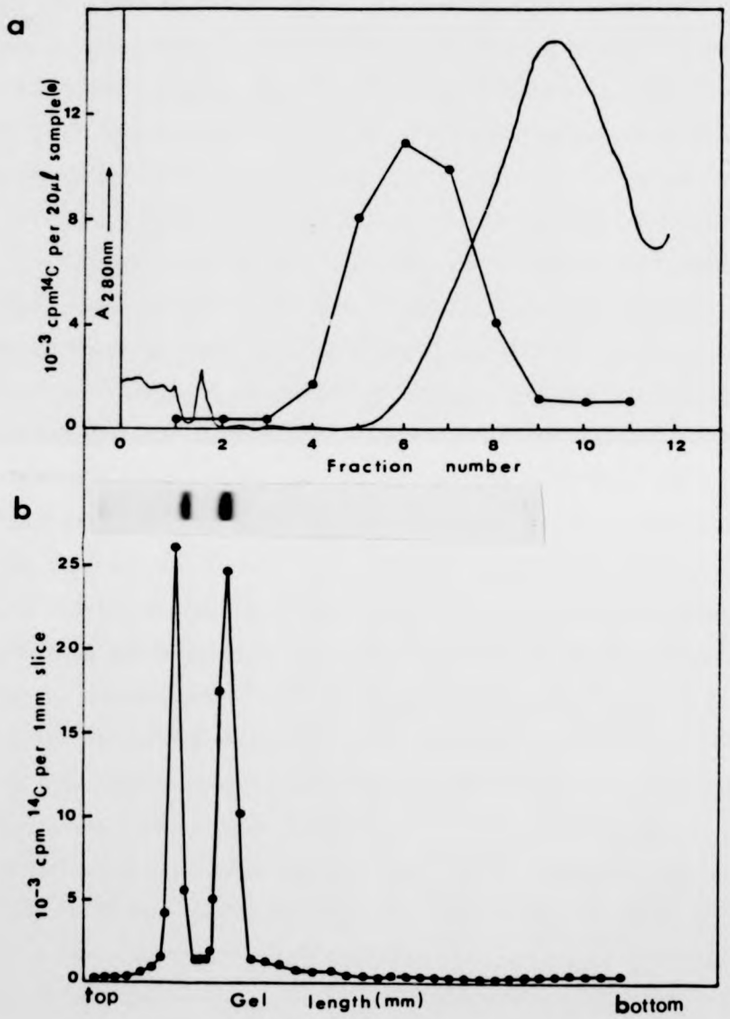


Figure 3.6 Isolation of RuBisCO from Rhodopseudomonas sphaeroides by sucrose gradient centrifugation

a) A_{280} nm of the soluble protein extract of R. sphaeroides (grown on butyrate-bicarbonate medium) centrifuged into 0.2 to 0.8 M step sucrose gradient. The RuBP dependent $^{14}CO_2$ fixing activity of the 1 ml fractions is superimposed.

b) Non-denaturing polyacrylamide gel electrophoresis at pH 7.5 of the pooled active RuBisCO fractions from the sucrose gradient centrifugation shown above. Gels were in duplicate: one which was stained for protein and the other sliced into 1 mm length and each slice assayed for RuBP dependent $^{14}CO_2$ fixing activity. Gels were polymerised using 5% (w/v) acrylamide.

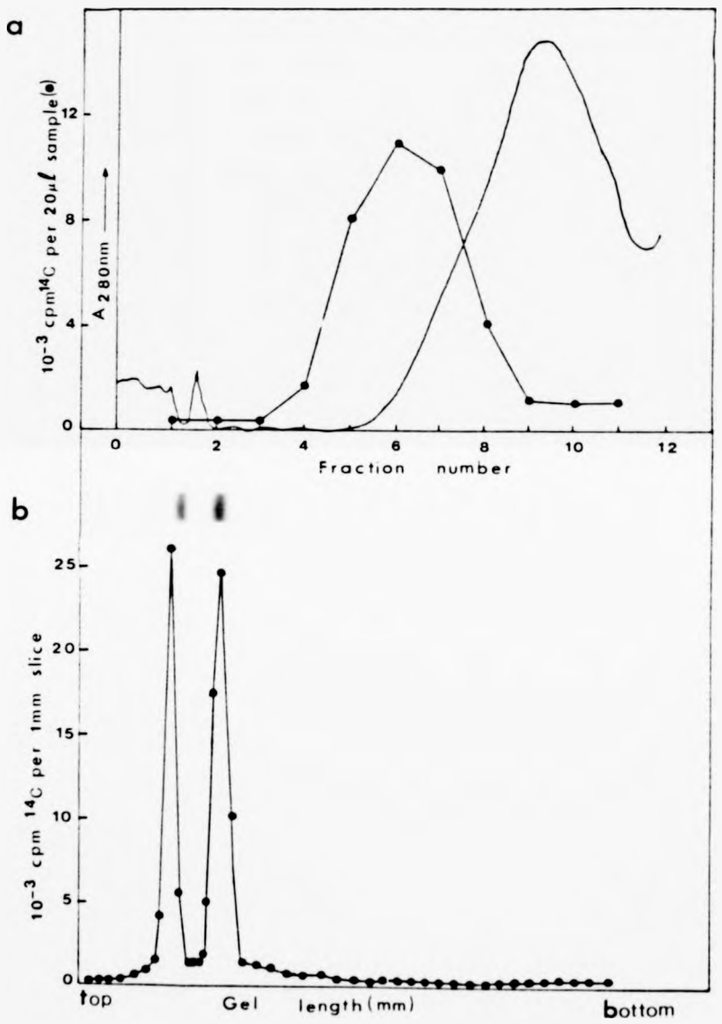


Figure 3.6 Isolation of RuBisCO from Rhodopseudomonas sphaeroides by sucrose gradient centrifugation

a) A_{280} nm of the soluble protein extract of R. sphaeroides (grown on butyrate-bicarbonate medium) centrifuged into 0.2 to 0.8 M step sucrose gradient. The RuBP dependent $^{14}CO_2$ fixing activity of the 1 ml fractions is superimposed.

b) Non-denaturing polyacrylamide gel electrophoresis at pH 7.5 of the pooled active RuBisCO fractions from the sucrose gradient centrifugation shown above. Gels were in duplicate: one which was stained for protein and the other sliced into 1 mm length and each slice assayed for RuBP dependent $^{14}CO_2$ fixing activity. Gels were polymerised using 5% (w/v) acrylamide.

enzymes to homogeneity. However, the purification procedure using step sucrose gradient centrifugation proved useful for the isolation of both the large and the intermediate molecular weight class of RuBisCO from R. sphaeroides in a single step.

c) Rhodopseudomonas palustris

Ribulose bisphosphate carboxylase/oxygenase from the soluble extract of R. palustris was easily separated from the bulk of the soluble protein after centrifugation into 0.2-0.8 M step sucrose gradient. Figure 3.7a shows the fractionation profile of the soluble protein extract of R. palustris centrifuged into a 0.2-0.8 M step sucrose gradient. Most of the 280 nm absorbing material is at the upper third of the gradient. The RuBP dependent CO_2 fixing activity of the 1 ml fractions shows that ribulose bisphosphate carboxylase sedimented more rapidly than the bulk of the UV-absorbing material in the gradient. The peak of enzyme activity was sharp as opposed to the broad peaks observed for R. blastica and R. sphaeroides under otherwise identical isolation procedure. The maximum peak of enzyme activity was in Fraction 5. Pooled active fractions (6 and 7) from the sucrose gradient centrifugation had a specific activity of 1.53 representing about 25-30 fold purification over the crude extracts. Moreover, the recovery of the active enzyme from fractions 5, 6 and 7 of the sucrose gradient centrifugation consistently approached 58% of the enzyme units initially applied to the gradient. The pooled active fractions of the isolated enzyme preparations was subjected to 5% (w/v) non-denaturing disc gel electrophoresis, and subsequently assayed for ribulose bisphosphate carboxylase as described previously. Figure 3.7b shows the electrophoretogram of enzyme preparations along with the activity in the gel slices. Two major stained protein bands were obtained on the gel. However, when the duplicate gel was sliced and

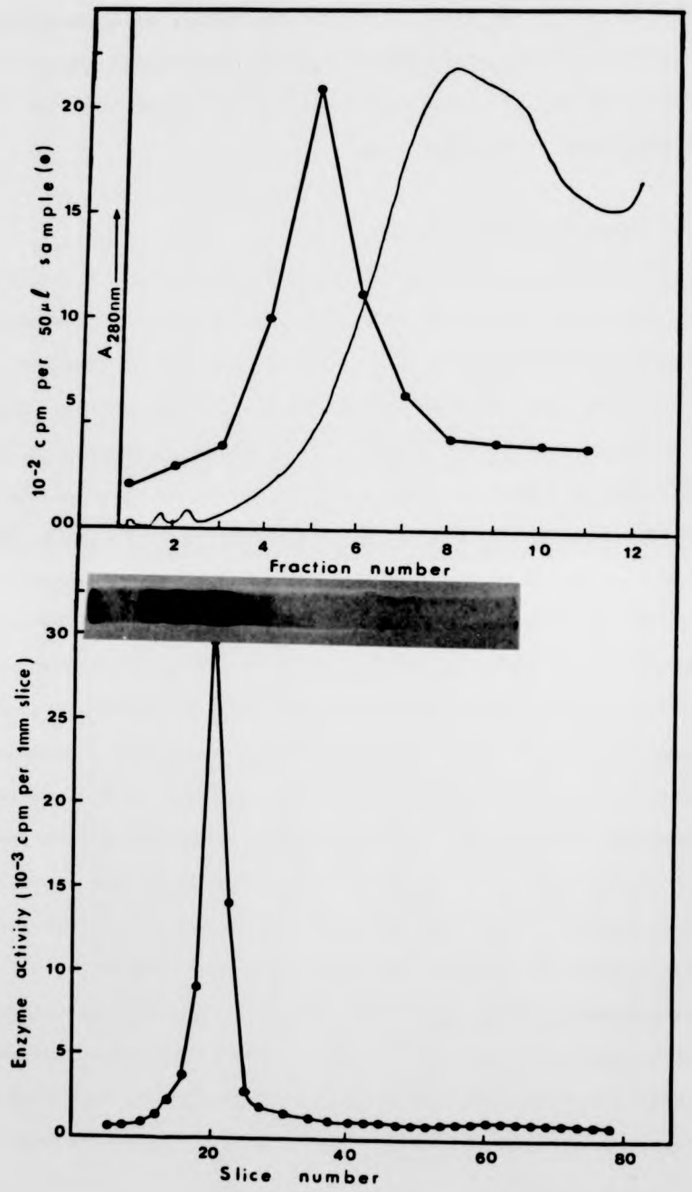


Figure 3.7 Partial purification of ribulose bisphosphate carboxylase/oxygenase from Rhodopseudomonas palustris

Soluble protein extract of R. palustris was centrifuged into 0.2 to 0.8 M step sucrose gradients. Active RuBisCO fractions were treated as described for Figure 3.6.

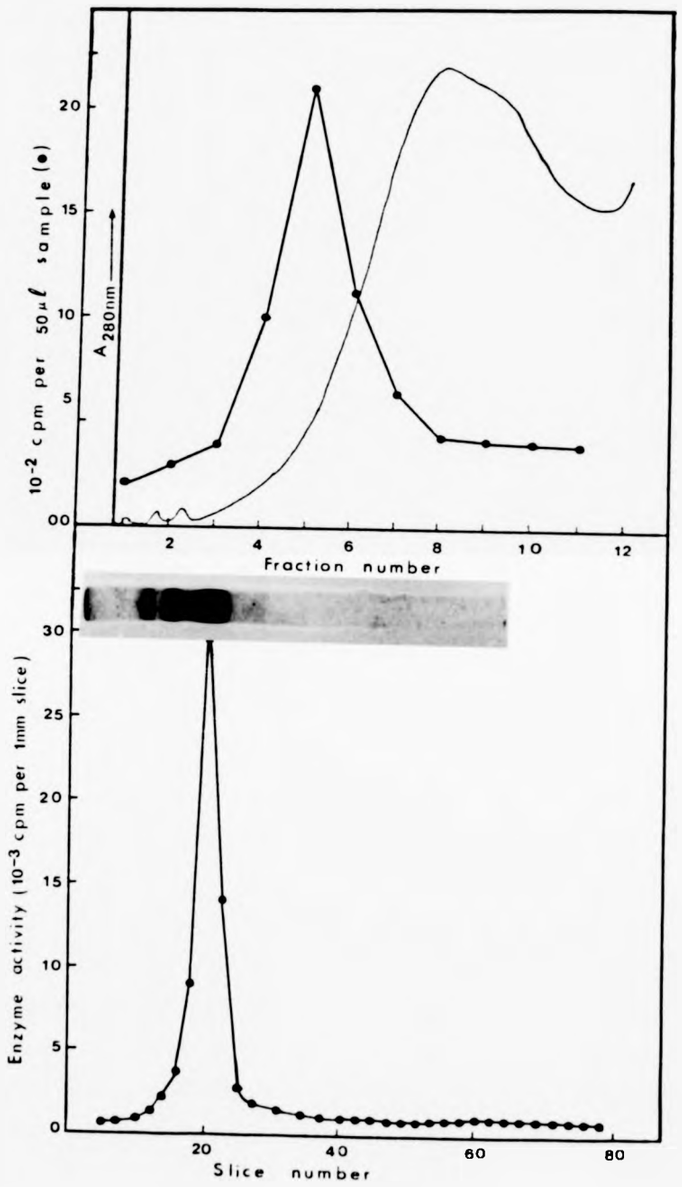


Figure 3.7 Partial purification of ribulose bisphosphate carboxylase/oxygenase from Rhodopseudomonas palustris

Soluble protein extract of R. palustris was centrifuged into 0.2 to 0.8 M step sucrose gradients. Active RuBisCO fractions were treated as described for Figure 3.6.

each slice assayed for RuBP dependent $^{14}CO_2$ fixing activity, only one peak of enzyme activity, which correspond to one of the stained bands was obtained (Figure 3.7c). Furthermore, the peak of enzyme activity in the gel slices corresponds to the position of the slower migrating (large molecular weight) form of the RuBisCO enzyme from R. sphaeroides when analysed under the same conditions. Presumably, the ribulose bisphosphate carboxylase/oxygenase from Rhodopseudomonas palustris is of large molecular weight class. A homogeneous RuBisCO preparation from R. palustris is needed to confirm this speculation. However, the results presented herein show that R. palustris synthesises only one form of ribulose bisphosphate carboxylase/oxygenase when grown photoheterotrophically either on butyrate- bicarbonate or pyruvate-malate medium.

3.7 Partial Purification of Ribulose Bisphosphate Carboxylase/Oxygenase from Rhodomicrobium vannielii

Ribulose bisphosphate carboxylase/oxygenase (RuBisCO) has been previously purified from R. vannielii and was shown to be unstable (Taylor and Dow, 1980). Batch cultures of R. vannielii strain (Rm5) undergo complex cell cycle in which three different cell types exist (Dow, 1974; Whittenbury and Dow, 1977; Dow et al., 1983). However, it is not known whether the different cell types, i.e. the swarmer, the chain and the exospore cells synthesise different molecular forms of RuBisCO. The one step isolation procedure using step sucrose gradient was therefore used to purify the enzyme from heterogeneous culture of R. vannielii. The enzyme preparations were also subjected to the in situ polyacrylamide gel electrophoresis with a view to screening for the presence of one or more different molecular forms of the enzyme in R. vannielii.

Figure 3.8 shows the absorbance trace at 280 nm of the soluble extract of R. vannielii centrifuged into a 0.2 to 0.8 M step sucrose gradient upon which the RuBP dependent CO_2 fixing activity of the 1 ml fractions is superimposed. The RuBisCO peak fractions sedimented faster, and was separated from the bulk of the soluble protein. To examine the purity of the enzyme preparation, fractions of high RuBisCO activity were subjected to SDS polyacrylamide gel electrophoresis. Figure 3.9 shows the electrophoretogram of the coomassie stained gel. The peak fraction was largely composed of one detectable polypeptide in the gel (Figure 3.9, lanes 6 and 7). The approximate molecular weight of this polypeptide, calculated from its electrophoretic mobility relative to marker proteins, was 56,000. This polypeptide probably corresponds to the large subunit of the RuBisCO enzyme from R. vannielii.

Although the small subunit of the enzyme was not stained in this gel, it could be possible that it ran with the dye front, thereby being eluted from the gel during electrophoresis. Nevertheless, the results show it is possible to purify ribulose bisphosphate carboxylase/oxygenase from R. vannielii to a virtual homogeneity in a single step by centrifugation of the soluble extract into step sucrose gradient for only 2.5h using a fixed angle head rotor.

To determine whether there is more than one molecular form of RuBisCO in R. vannielii (Rm5), the $^{14}CO_2$ fixing fractions from the sucrose gradient were pooled and electrophoresed on 5% non-denaturing tube gels. One of the gels was stained for protein and the other was sliced into 1 mm and each slice assayed for RuBP dependent $^{14}CO_2$ fixing activity. Figure 3.10 shows the electrophoretogram of the stained gel along with the profile of enzyme activity in the gel slices. A densitometric scan at A_{540} of the

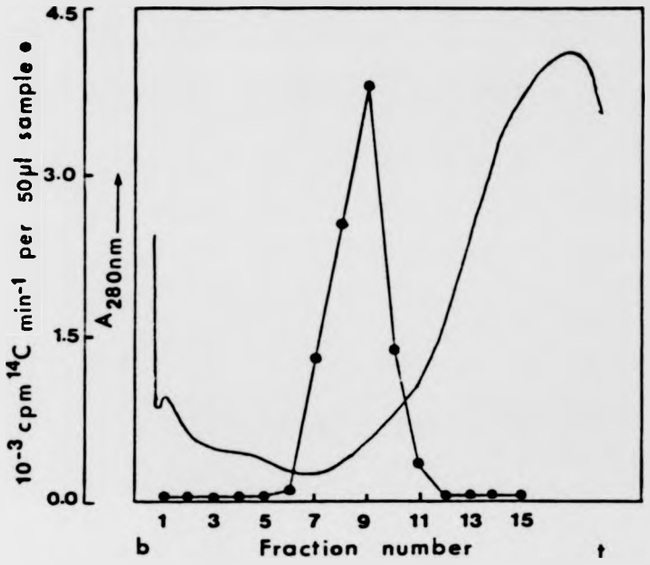


Figure 3.8 A_{280} nm of the soluble protein extract from Rhodomicrobium vannielii (Rm5) centrifuged into 0.2 to 0.8 M step sucrose gradients

The RuBP dependent $^{14}CO_2$ fixing activity (cpm/50 µl sample) of the 1 ml fractions is superimposed. (b) = bottom of the gradient and (t) = the top.

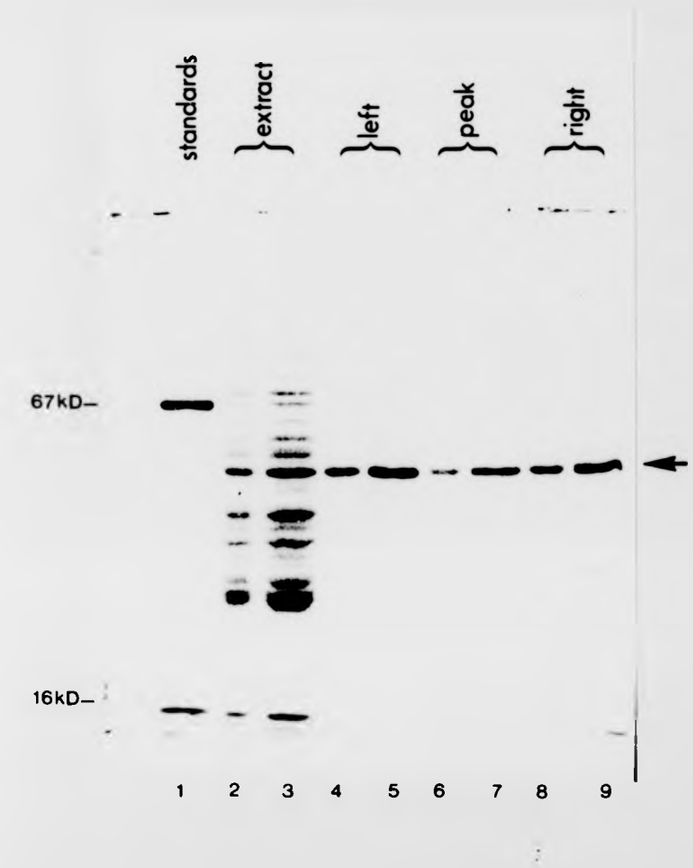
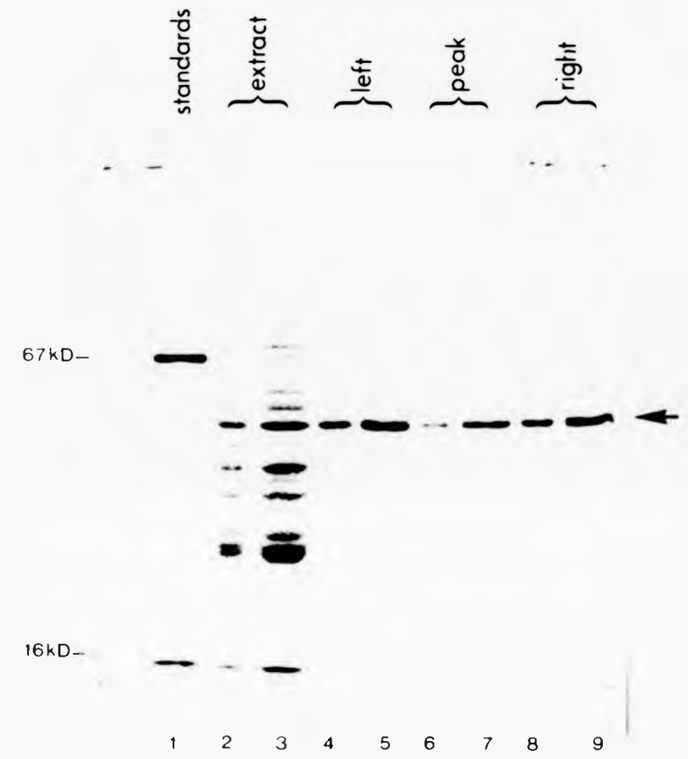


Figure 3.9 Sodium dodecyl sulphate slab gel electrophoresis of the R. vannielii RuBisCO peak fractions from the sucrose gradient centrifugation shown in Figure 3.8

Gel was polymerised using 10% (w/v) acrylamide and stained with Coomassie blue. The arrow indicates the large subunit polypeptide of the RuBisCO enzyme.



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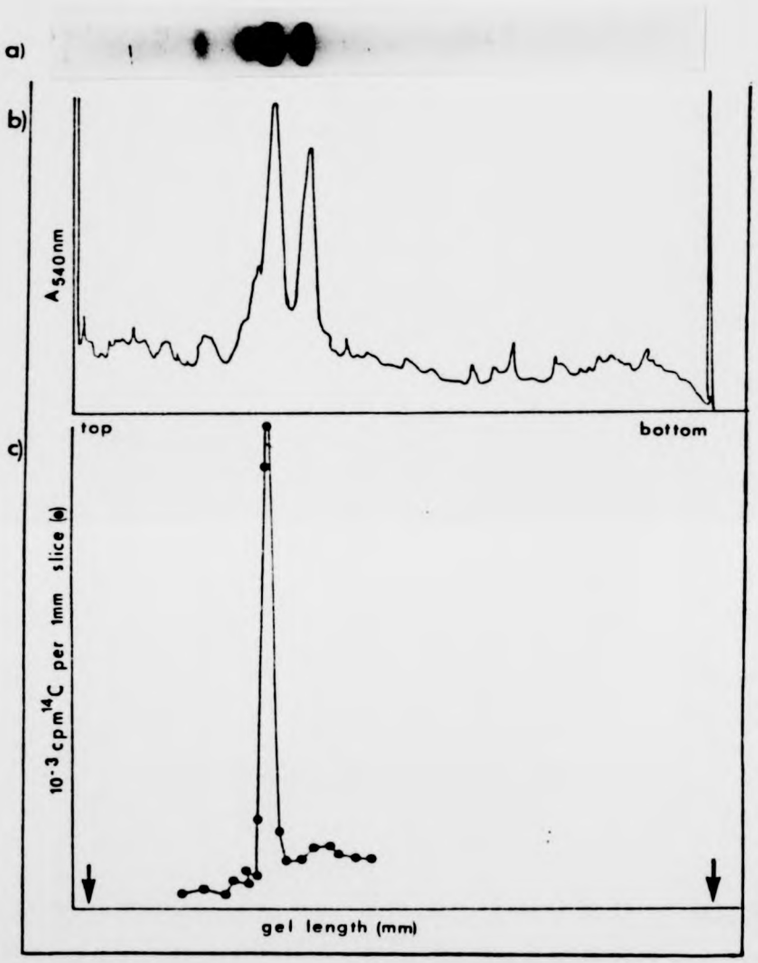
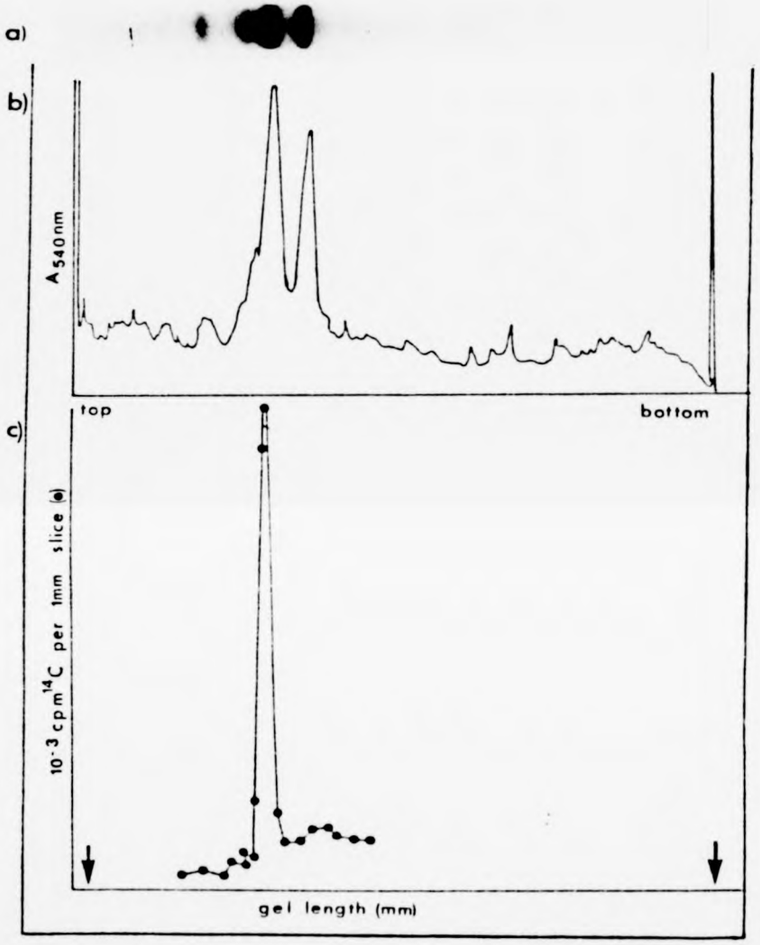


Figure 3.10 Non-denaturing polyacrylamide gel electrophoresis of the pooled R. vannielii RuBisCO fractions from the sucrose gradient centrifugation shown in Figure 3.8

a) Coomassie stained gel.

b) Densitometric scan (at 540 nm) of the gel above (a).

c) RuBP dependent $^{14}CO_2$ fixing activity of the 1 mm gel slices per 15 min assay.



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a) Coomassie stained gel.

b) Densitometric scan (at 540 nm) of the gel above (a).

c) RuBP dependent $^{14}CO_2$ fixing activity of the 1 mm gel slices per 15 min assay.

stained gel showed two major protein bands, but clearly only one peak of enzymic activity was present in the gel (Figure 3.10c). The peak of enzymic activity correspond to one of the stained bands. From this study it is concluded that heterogeneous culture of R. vannielii synthesises only one molecular form of RuBisCO when this organism is grown photoheterotrophically either on pyruvate-malate or butyrate-bicarbonate medium.

3.8 Discussion on the Isolation of Bacterial RuBisCO by sucrose gradient centrifugation

The sucrose gradient centrifugation method developed for the isolation of RuBisCO from the photosynthetic prokaryotes in mild and require small samples to be analysed. Centrifugation in fixed angle rotor requires a short time to run (2.5h) as opposed to long hours (15 h) when swing out rotors are used (Tabita and McFadden, 1974c), thereby reducing the possibility of loss of enzyme activity or subunits from the RuBisCO enzyme during lengthy purification procedures. Moreover, the method allows for eight samples to be run at a time. It is therefore possible to make a qualitative comparison between the RuBisCO enzyme from a number of bacterial species under the same isolation conditions. However, step sucrose as opposed to linear sucrose gradient was found to give a better resolution in this isolation technique. Because of the relatively short time required for the centrifugation procedure, this method may also be useful for the isolation of biological materials which show tendency to loose activity during lengthy purification procedures.

The in situ assay method developed for the detection of ribulose

bisphosphate carboxylase activity in gel slices is more sensitive than the coomassie blue staining technique. The step sucrose gradient isolation procedure coupled with a non-denaturing polyacrylamide gel assay for RuBisCO was used to detect the presence of two different molecular forms of this enzyme in extracts of R. blastica grown phototrophically on pyruvate-malate medium. The presence of two different molecular forms of RuBisCO in this organism has hitherto not been reported. Similarly, this isolation technique was used to show the presence of two different molecular forms of RuBisCO in the extract of R. sphaeroides as earlier reported by Gibson and Tabita (1977a). These observations therefore, further strengthened the earlier report by Eckersley and Dow (1980) of the close physiological relatedness between R. blastica and R. sphaeroides/R. capsulata.

It is also shown in this study, that heterogenous cultures of R. vannielii (Rm5) and R. palustris synthesise only one form of RuBisCO when these organisms were grown in batch on either butyrate-bicarbonate or pyruvate-malate media. R. vannielii exhibits a polymorphic cell cycle in which three distinct cell types are expressed during growth, while R. palustris exhibits a dimorphic cell cycle (Section 3.1). The findings that these two organisms synthesise one molecular form of RuBisCO suggest that the dual enzyme system (i.e. different molecular forms of RuBisCO as found in R. blastica, R. sphaeroides and R. capsulata), is not a consequence of dimorphism or different cell types. The two enzyme concept may be an inherent physiological property of the organisms in which different molecular forms of RuBisCO are found. Therefore, the physiological significance of the presence of different molecular forms of this enzyme in R. blastica needs further investigations.

The sucrose gradient centrifugation procedure was found not to be suitable for the isolation of the RuBisCO enzyme from Rhodospirillum rubrum. On sucrose gradient centrifugation, the enzyme from R. rubrum sedimented with the bulk of the soluble protein the extract. This is presumeably due to the low molecular weight nature of the RuBisCO (MW - 114,000, Tabita and McFadden, 1974a) from this organism. However, this method can be used to isolate the RuBisCO enzyme from Pseudomonas oxalaticus (Section 3.6.2), which is reported to possess an intermediate size molecular weight enzyme (Lawlis et al., 1979).

In conclusion, the technique of sucrose gradient centrifugation for the isolation of RuBisCO coupled with non-denaturing polyacrylamide gel assays for this enzyme have a unique usefulness in that they can be used to study the physiology of synthesis of the two forms of the RuBisCO enzyme in the same organism. The enzymes can both be isolated under the same conditions (within short time) and their relative levels (in terms of activity and quantity) compared in the same gel system. Furthermore, the method would afford rapid isolation of the enzyme from numerous sources; the advantage of this will be to characterize the enzyme activity with respect to carboxylation and oxygenation of RuBP, especially where stability is a problem. As appropriate, simple purification steps such as ammonium sulphate precipitation may be added to the step sucrose gradient centrifugation to ensure rapid purification of the enzyme to homogeneity. This method may be useful for the estimation of in vivo levels of RuBisCO protein in physiological studies, and for the preparation of small amounts of the enzyme for in vitro studies of enzyme structure and function.

Chapter 4

Results and Discussion II

Purification and Properties of Ribulose-1,5-bisphosphate Carboxylase/Oxygenase from *Rhodopseudomonas blastica*

4.1 Purification of two different molecular forms of Ribulose bisphosphate carboxylase-oxygenase from Rhodopseudomonas blastica

The single step purification procedure using sucrose gradient centrifugation and subsequent non-denaturing polyacrylamide gel analysis of the enzyme preparations shows the presence of two forms of ribulose bisphosphate carboxylase-oxygenase (RuBisCO) in the soluble extracts of R. blastica. However, this method could not be used to obtain electrophoretically pure enzymes (Section 3.5.3a). A conventional purification procedure was therefore adopted for the purification of the RuBisCO enzymes from R. blastica. the protocol for which is described in the materials and methods section (2.19).

Soluble protein extracts were prepared from 200 g wet weight of cells grown photoheterotrophically under anaerobic conditions either on pyruvate-malate or butyrate-bicarbonate medium. Soluble protein extracts were enriched 9-fold for RuBisCO by precipitation with 60% (w/v) ammonium sulphate saturation (Table 4.1). Enzyme eluted from a Bio-gel A-5M column as two diffuse peaks of RuBP dependent $^{14}CO_2$ fixing activity (Figure 4.1). The peaks were separated from the bulk of the soluble protein and pigments present in the ammonium sulphate precipitate. The pooled active frations from the Bio-gel column were loaded onto a DEAE-Trisacryl ion exchange chromatography column and eluted with a 0.01 to 0.2 M linear gradient of NaCl in TEMMB buffer. Two distinct peaks of ribulose bisphosphate (RuBP) dependent carboxylase activity eluted from the DEAE-Trisacryl column (Figure 4.2). Two peaks of the enzyme activity were always obtained whether or not, extracts were pre-treated with PMSF, a protease inhibitor, before the purification procedures. Enzyme from the first peak emerging after DEAE-Trisacryl chromatography will be

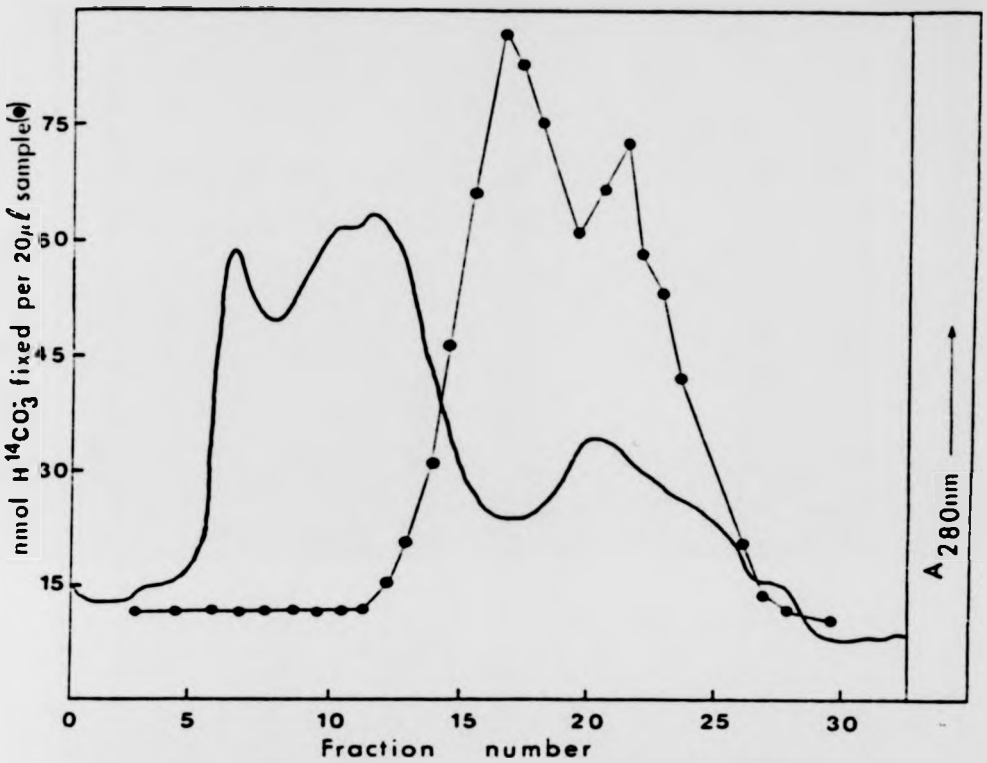


Figure 4.1 Bio-gel A-5M column chromatography of the ammonium sulphate fractionation of the soluble protein extract of Rhodopseudomonas blastica

Enzyme was eluted with TEMMB buffer.

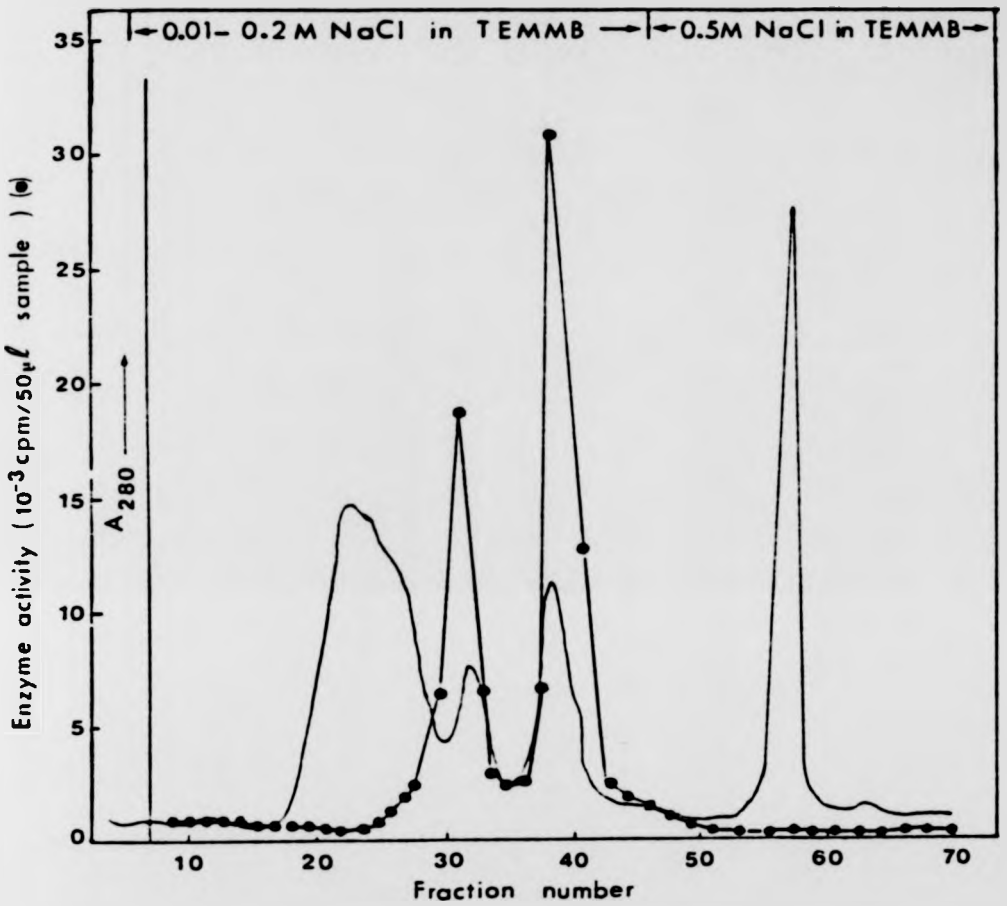


Figure 4.2 DEAE-Trisacryl column chromatography of RuBisCO from Rhodopseudomonas blastica grown photoheterotrophically on pyruvate-malate medium

RuBP carboxylase activity (●) is expressed as cpm of [^{14}C] bicarbonate fixed during a 2 min assay of a 50 µl sample from each fraction of 5 ml in the standard assay.

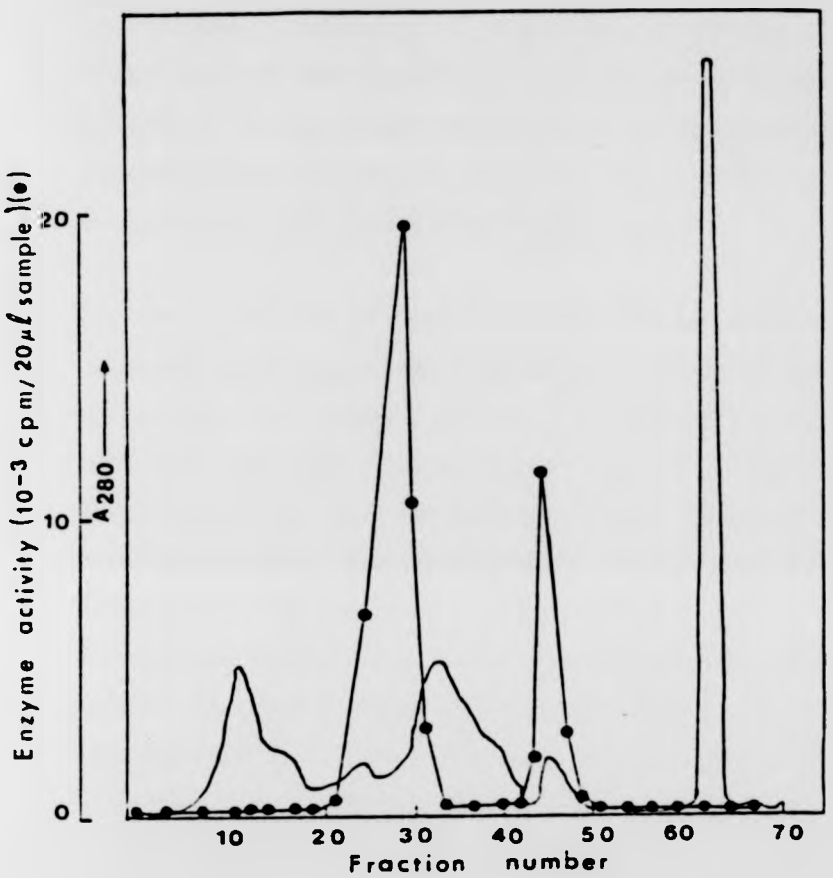


Figure 4.3 DEAE-Trisacryl column chromatography of RuBisCO from Rhodopseudomonas blastica grown photoheterotrophically on butyrate-bicarbonate medium

Assay conditions were as for Figure 4.2

referred to as the Form I ribulose bisphosphate carboxylase/oxygenase, and the Form II refers to the second peak of activity. However, enzymes eluted from the DEAE column show (in terms of activity) that the Form II RuBisCO is the predominant form in cells grown photoheterotrophically on pyruvate-malate (Figure 4.2) while the Form I predominate in cells grown on butyrate-bicarbonate medium (Figure 4.3).

The peak I and peak II enzymes from DEAE-Trisacryl column were pooled separately and concentrated. These concentrated samples were subsequently centrifuged into 0.2-0.8 M step sucrose gradients as previously described (Section 2.17). Figure 4.4 shows the A_{280}nm of the 1 ml fractions of the Form I and the Form II enzymes centrifuged into sucrose gradients. The RuBP dependent $^{14}CO_2$ fixing activity is superimposed. In each case a symmetrical sharp peak of $^{14}CO_2$ fixing activity was associated with each preparation after centrifugation. However, the Form I enzyme activity peak (Figure 4.4a) sedimented faster than the Form II (Figure 4.4b) under otherwise, identical centrifugation procedures. This indicates that the Form I enzyme may well have a higher molecular weight than the Form II enzyme.

The purity of the enzyme preparations was checked by running samples of the Form I and the Form II enzymes from sucrose gradients on 5% (w/v) non-denaturing polyacrylamide gels. Gels were run in duplicate, one was stained for protein and the other subjected to <u>in situ</u> polyacrylamide gel assay for ribulose bisphosphate carboxylase activity. Figure 4.5 shows the electrophoretograms of the Form I and Form II RuBisCOs along with the corresponding activity profiles in gel slices. In each case, one major peak of enzyme activity is associated with a single stained protein band. This indicates that the purified enzymes are free from contamination, and

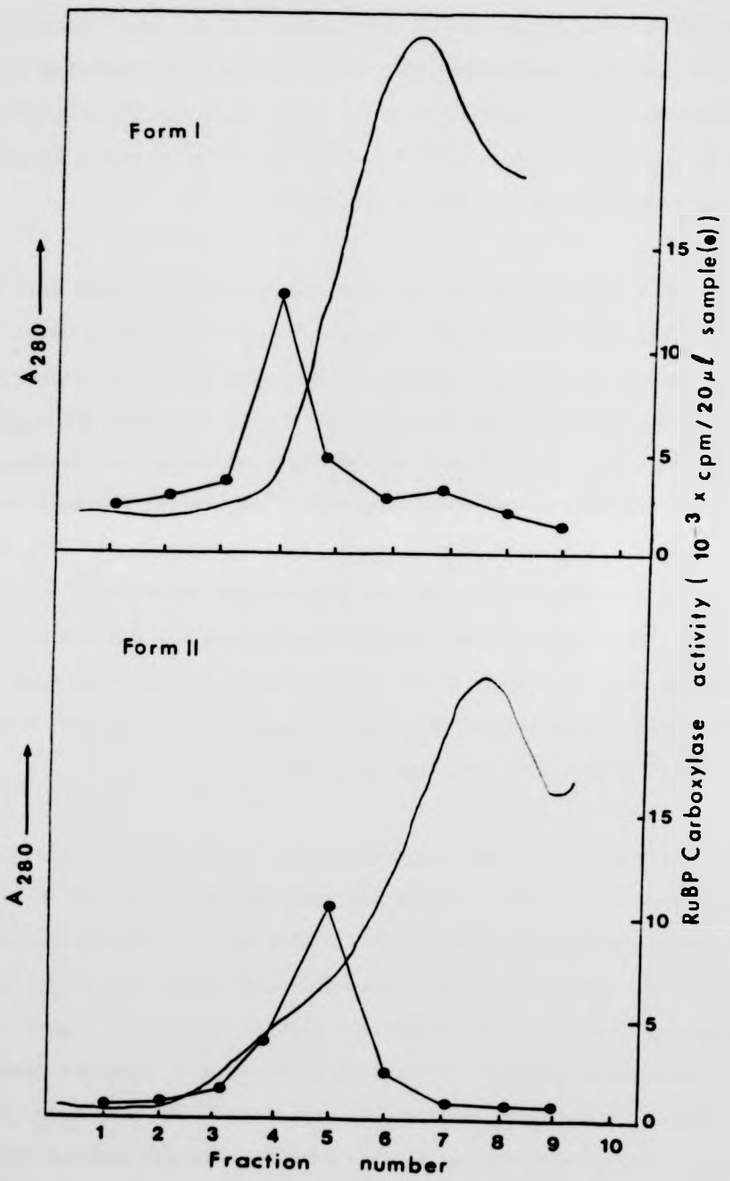


Figure 4.4 A_{280} nm of the two peak fractions of RuBisCO activity (obtained from the DEAE column of R. blastica) centrifuged into 0.2 to 0.8 M step sucrose gradients

RuBP dependent $^{14}CO_2$ fixing activity (cpm/2 min assay of 20 µl sample) of the 1 ml fractions (●) is superimposed. Form I refers to the Peak I and Form II to Peak II fractions from the DEAE-trisacryl column.

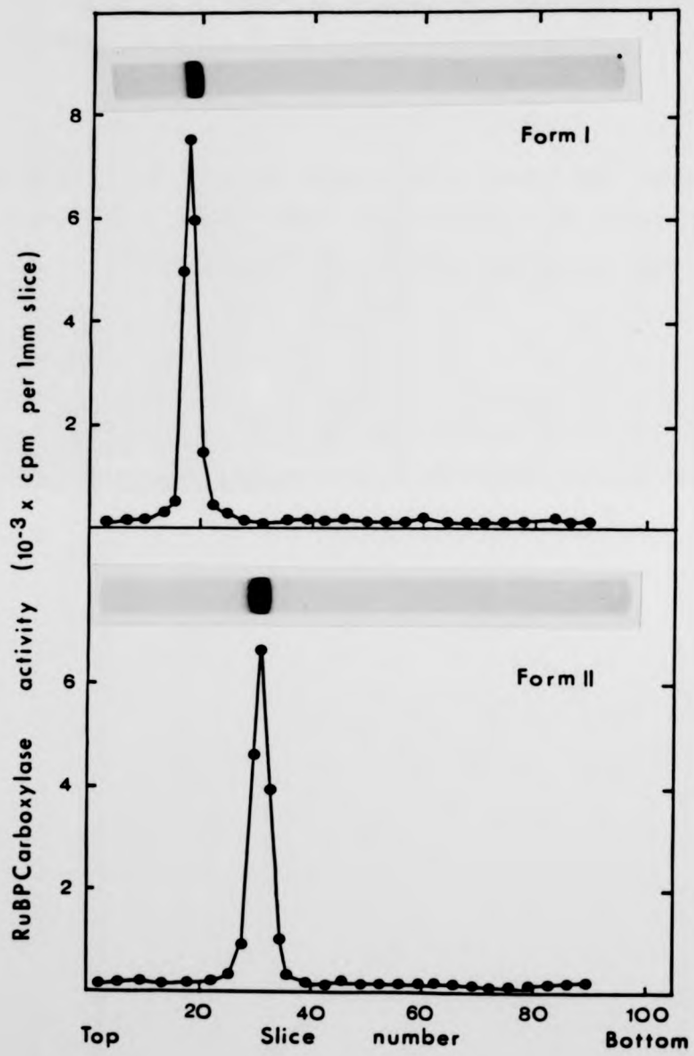
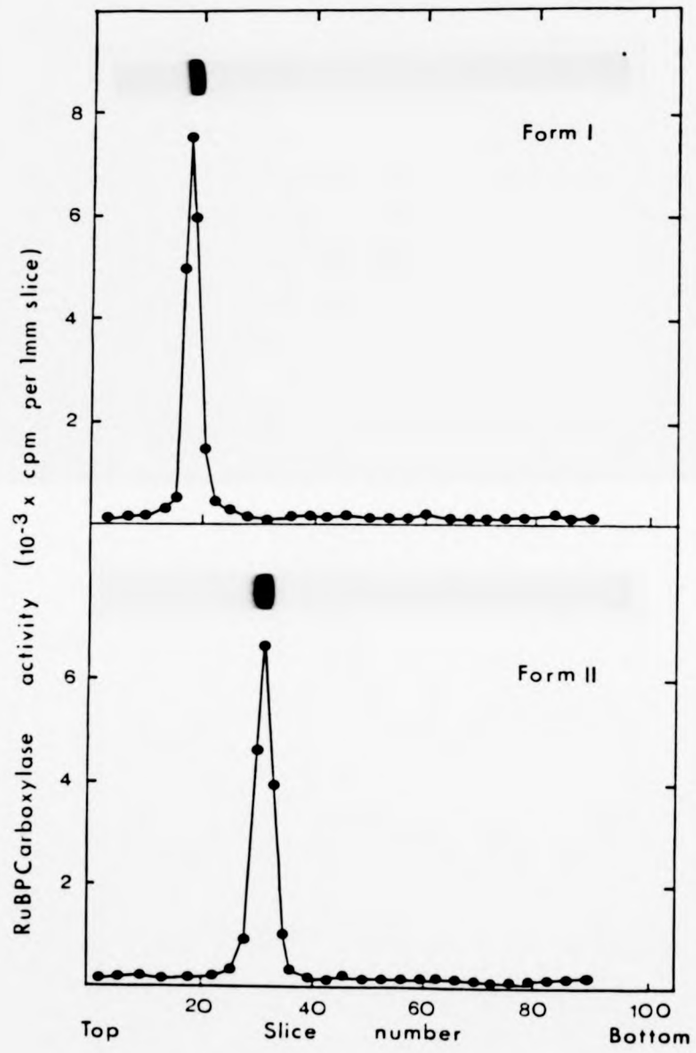


Figure 4.5 Non-denaturing polyacrylamide gel electrophoresis of the Form I and Form II RuBisCO fractions from sucrose gradient centrifugation as shown in Figure 4.4

In each case 30 ug of protein was applied to each gel polymerized from 5% (w/v) acrylamide. Gels were run at pH 8.8. Conditions of staining and *in situ* polyacrylamide gel assay for RuBP carboxylase are as described in materials and methods (Section 2.18).



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that each enzyme preparation is homogeneous.

The possibility of the two molecular forms of the RuBisCO purified from R. blastica being size or charge isomers was investigated. The purified enzymes were subjected to electrophoresis on non-denaturing polyacrylamide rod gels polymerised from 3 to 6% (w/v) acrylamide. Figure 4.6a shows the electrophoretogram of the migration patterns of the purified enzymes at varying gel concentrations along with the corresponding Hedrick and Smith' plots (Hedrick and Smith, 1968). The plots of log Rm against gel concentrations for the two enzymes have different slopes and do not extrapolate to a common origin (Figure 4.6b). This indicates that the two forms of the RuBisCO enzyme are neither size nor charge isomers.

A summary of the protocol for the purification of RuBisCO from Rhodopseudomonas blastica is shown in Table 4.1. For comparative purposes, the specific activity and the yield of the enzymes purified from cells grown on butyrate-bicarbonate medium have been included. The final specific activity of the Form I and Form II enzymes from pyruvate-malate grown cells were 2.53 and 1.62 respectively. A 3.2 to 4.8% enzyme recovery was routinely achieved. Assuming that no inhibitors or activators of these enzymes were removed during purification, then taken together, the Form I and Form II enzymes represent about 0.9 to 1.4% of the total soluble protein in the 120,000 x g supernatant of the pyruvate-malate grown cells. On the other hand, purified enzymes from butyrate-bicarbonate grown cells have final specific activities of 2.61 and 1.78 for the Form I and Form II respectively and a 2.6 to 4.4% yield. This shows that the two enzymes represent about 6.4 to 9.4% of the total soluble protein of the butyrate-bicarbonate grown cells. These results

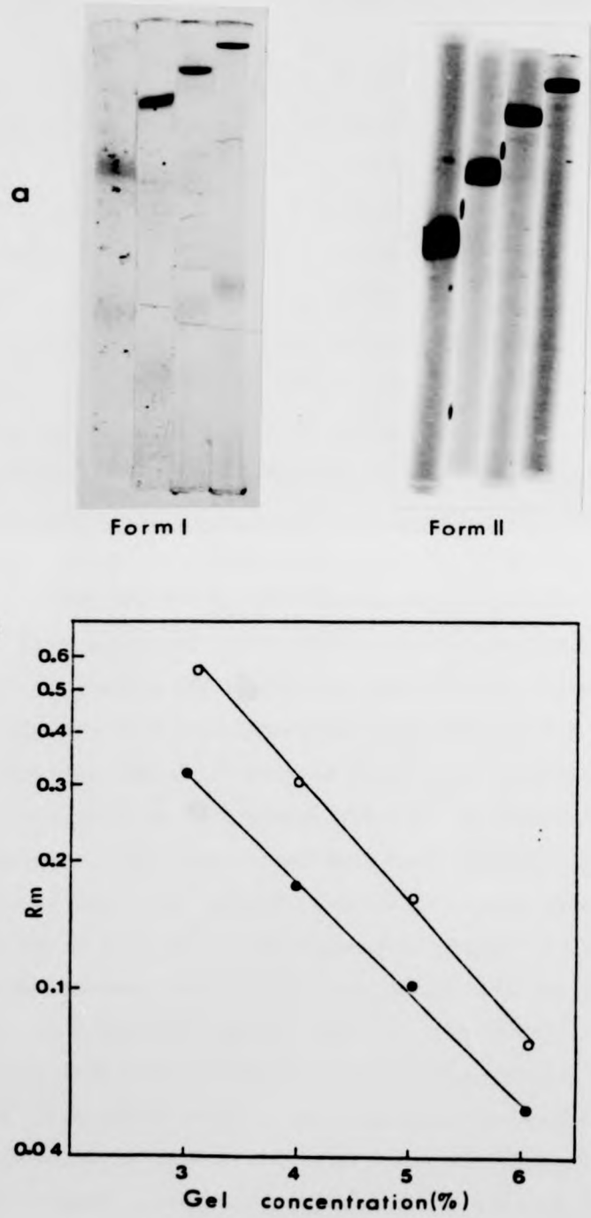
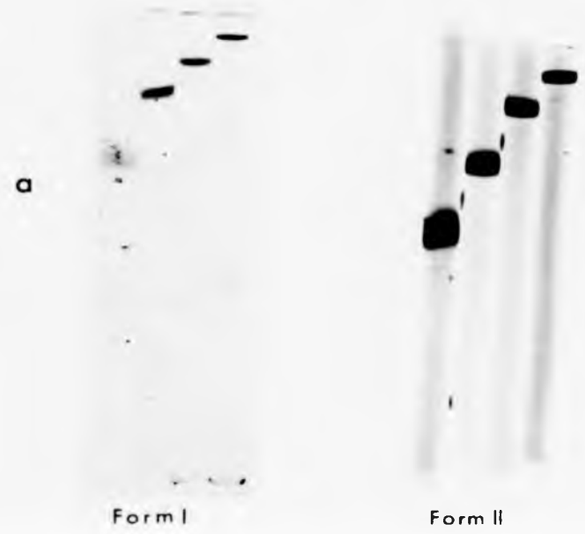
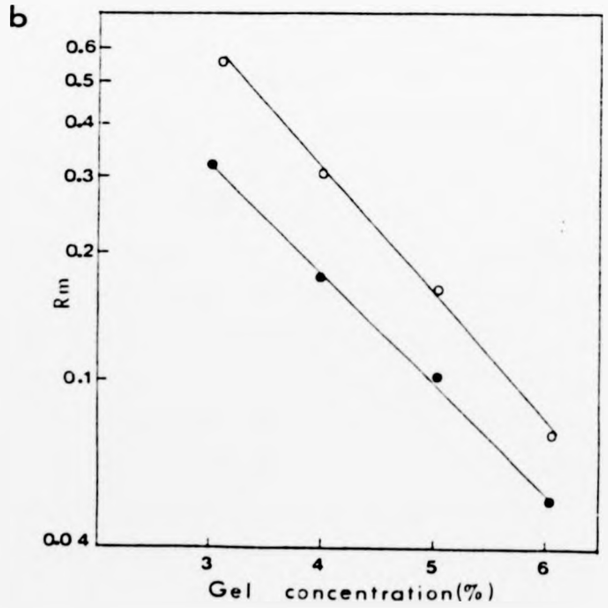


Figure 4.6 Polyacrylamide gel electrophoresis of the homogeneous Form I and Form II RuBisCOs from R. blastica

a) In each case, gels were polymerised from (left to right) 3, 4, 5 and 6% (w/v) acrylamide.

b) Plot of log Rm values (from (a)) of the Form I and Form II enzymes against gel concentration (Hedrick plot, Hedrick and Smith (1968).
● Form I
O Form II





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a) In each case, gels were polymerised from (left to right) 3, 4, 5 and 6% (w/v) acrylamide.

b) Plot of log Rm values (from (a)) of the Form I and Form II enzymes against gel concentration (Hedrick plot, Hedrick and Smith (1968).
- ● Form I
- O Form II

Table 4.1 Isolation of ribulose bisphosphate carboxylase/oxygenase from Rhodopseudomonas blastica

Purification step	Protein (mg)	Total activity (units)	Specific activity (units/mg protein)	Recovery (%)
120,000xg supernatant	3087	71.05	0.023 (0.168)+	100
1 M $MgCl_2$ treatment	1079	61.7	0.057	87
60% (w/v) ammonium sulphate treatment	307	58.3	0.190	82
Gel filtration on Biogel A-5M	55.2	26.5	0.480	37
Peak I DEAE-Trisacryl eluates (pooled)	3.8	5.39	1.42	7.6
Peak II DEAE-Trisacryl eluates (pooled)	9.6	8.41	0.875	12
0.2-0.8 M step sucrose gradient Peak I	0.91	2.3	2.53 (2.61)+	3.2 (4.4)+
0.2-0.8 M step sucrose gradient Peak II	2.10	3.4	1.62 (1.78)+	4.8 (2.61)+

+ Data obtained from cells grown on butyrate-HCO_3 medium.

are comparable to the observations that phototrophic cells of R. sphaeroides grown on butyrate contain 12-fold more enzyme when compared to cells grown on malate (Gibson and Tabita, 1977a).

The absorbance ratio at 280/260 nm of the purified enzymes at concentrations of 1.0 $mg.ml^{-1}$ (1.0 cm path length) was 1.68 and 1.71 for the Form I and Form II enzymes respectively, thus indicating that the purified enzyme preparations were not contaminated by nucleic acid. The properties of the homogeneous enzymes were therefore investigated.

4.2 Molecular Properties of the Purified Ribulose Bisphosphate Carboxylase/Oxygenase from Rhodopseudomonas blastica

4.2.1 Stability of the Purified Enzymes

The stabilities of the two forms of RuBisCO purified from R. blastica were studied by measuring enzyme activity with time after storage at different temperatures. The purified enzymes are stable for at least one month when stored, sterile at -20°C at approximately 1 to 2 $mg.ml^{-1}$ in 0.5 M sucrose in TEMMB buffer. After storage at 2°C, the Form II enzyme lost about 2% of its original activity after 2 weeks, but the Form I enzyme did not show any loss in activity for the same period of storage. The purified enzymes could also be stored as pellets at -20°C after ammonium sulphate precipitation, for at least one month without any loss in enzyme activity. The results therefore, show that there was probably no substantial loss in activity with time during the purification procedures. This observation is consistent with those reported for ribulose bisphosphate carboxylase-oxygenase from Rhodopseudomonas sphaeroides (Gibson and Tabita, 1977a), Pseudomonas oxalaticus (Lawlis et

al., 1979) and Thiobacillus intermedius (Purohit et al., 1976a) but differes from that reported for enzyme from Rhodomicrobium vannielii which was unstable (losing about 50% of its maximal activity) when stored at -21°C for 21 days (Taylor and Dow, 1980).

4.2.2 Molecular Weights

The holoenzyme molecular weights of the purified enzymes were determined by gel filtration and on non-denaturing polyacrylamide disc gels, as described under materials and methods (2.21). Figure 4.7 shows the plot of the Kav (calculated from the elution volumes) against molecular weight of standard proteins from which the molecular weights of the Form I and Form II enzymes were determined using their Kav values. The molecular weights of the Form I and Form II enzyme as determined by gel filtration were 550,000 and 350,000 respectively. The molecular weights of the Form I and Form II enzymes were also determined from Hedrick plots by comparing their slopes (obtained from a plot of log Rm against gel concentration) with that of several protein standards. The Form I and Form II enzymes have molecular weight of 540,000 and 345,000 respectively as determined by the non-denaturing gel method. On the basis of the molecular weight classification (Anderson et al..1968). the purified Form I RuBisCO from R. blastica falls within the large molecular weight class (Mr = 500,000 to 600,000), while the Form II enzyme from the same organism is of the intermediate size class (Mr = 300,000 to 360,000). Certainly, further experimentation is required to rigorously confirm the molecular weights of these enzymes.

4.2.3 Quaternary Structure

The quaternary structure of the purified enzymes from R. blastica was determined after denaturation with SDS in the presence of

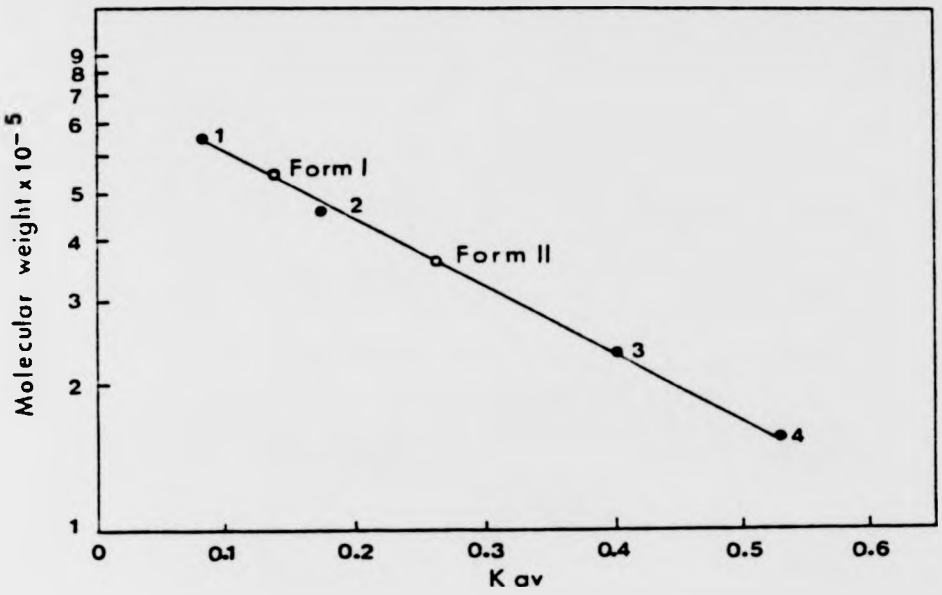


Figure 4.7 Estimation of the molecular weight of Rhodopseudomonas blastica RuBisCO on a sephadex G200 column chromatography

The calibration curve was obtained from the following protein standards of known molecular weights 1: thyroglobulin; 2: ferritin; 3: catalase; 4: aldolase.

$$Kav = \frac{Ve - Vo}{Vt - Vo}$$

where Ve = elution volume

Vt = bed volume

Vo = void volume

Figure 4.8 10% (w/v) SDS polyacrylamide gel electrophoresis of Form I and Form II RuBisCO from R. blastica

Gel was stained with silver nitrate.

Lane 1: pooled Peak I fractions from DEAE-trisacryl column.

Lane 2: pooled Peak II RuBisCO fractions from DEAE column.

Form 3: Form I enzyme after sucrose gradient centrifugation.

Lane 4: Form II enzyme after sucrose gradient centrifugation.

Figure 4.8 10% (w/v) SDS polyacrylamide gel electrophoresis of Form I and Form II RuBisCO from R. blastica

Gel was stained with silver nitrate.

Lane 1: pooled Peak I fractions from DEAE-trisacryl column.

Lane 2: pooled Peak II RuBisCO fractions from DEAE column.

Form 3: Form I enzyme after sucrose gradient centrifugation.

Lane 4: Form II enzyme after sucrose gradient centrifugation.

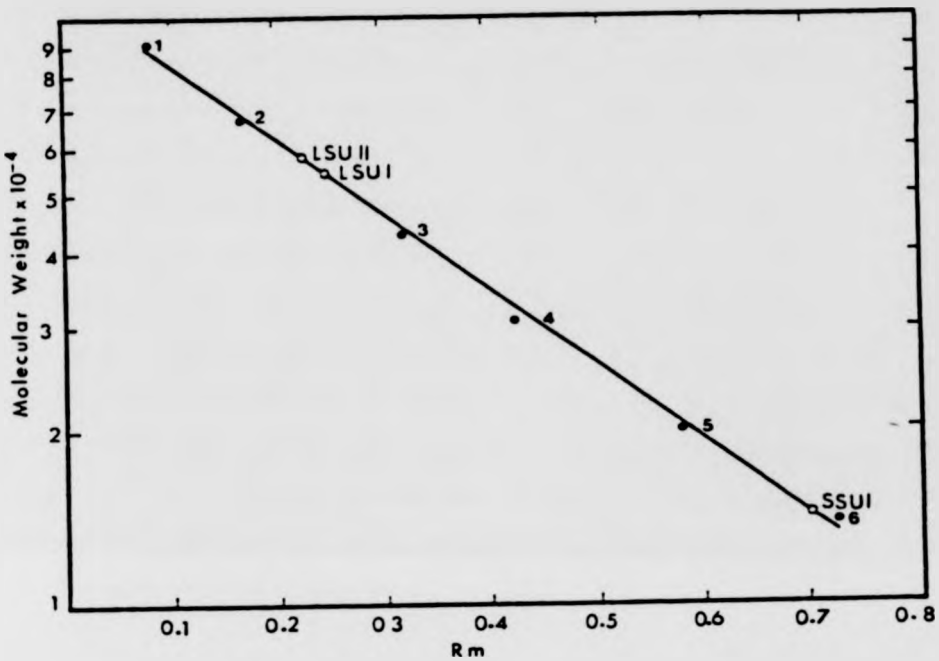


Figure 4.9 Estimation of the molecular weight of SDS-dissociated RuBisCO from R. blastica

The molecular weights of the subunits of the R. blastica enzymes were determined from a calibration curve obtained using the following standard proteins of known molecular weights:

1, phosphorylase b; 2, bovine serum albumin; 3, ovalbumin; 4, carbonic anhydrase; 5, trypsin inhibitor; 6, α-lactalbumin.

2-mercaptoethanol (Webber et al., 1972). The Form I enzyme was dissociated into two non-identical polypeptides as revealed by electrophoresis on SDS slab gels polymerized from either 10% (w/v) or exponential gradient of 10-30% (w/v) polyacrylamide (Figure 4.8). These polypeptides correspond to the large and the small subunits. Each of the subunits of the Form I enzyme is homogeneous as shown by the stained polypeptide bands on either gel system. The SDS slab gel analysis of the dissociated Form II enzyme shows that the enzyme is composed of only one polypeptide. This polypeptide was homogeneous as revealed by the SDS slab polyacrylamide gels and it migrated slower than the large subunit of the Form I RuBisCO from the same organism. There was no evidence for the presence of a small subunit in the Form II enzyme, whether gels were stained with Coomassie blue or by the more sensitive silver nitrate staining method of Wray (Wray et al., 1981).

The molecular weights of the purified enzymes from R. blastica were then determined by comparing their mobilities to several standards run on the same slab gel (Figure 4.9). The results show that the large subunit of the Form I enzyme has a molecular weight of 53,000 to 54,000 depending on the gel system used, while the small subunit has a molecular weight of approximately 13,500. The polypeptide obtained from the dissociation of the Form II enzyme has a molecular weight of 56,000 on 10% (w/v) SDS-polyacrylamide gels and 58,000 on 10-30% (w/v) polyacrylamide gels. These values are higher than those reported for the subunits of the two forms of RuBisCO purified from Rhodopseudomonas sphaeroides (Gibson and Tabita, 1977a).

Densitometric scans of a number of stained gels gave an average ratio of the large to small subunits of the Form I enzyme to be about 1:1. With a

molecular weight of approximately 550,000, the Form I RuBisCO from R. blastica is probably composed of eight large and eight small subunits, similar to the enzymes from eukaryotic sources (Kawashima and Wildman, 1970). Since the Form II enzyme is composed of only large subunits (Mr 58,000) and has a holoenzyme molecular weight of approximately 340,000, the enzyme is most probably composed of six large subunits. The quaternary structure of the RuBisCO enzyme from R. blastica is therefore, similar to that reported for the physiologically related organisms R. sphaeroides and Rhodopseudomonas capsulata (Gibson and Tabita, 1977a; Tabita, 1981).

4.3 Catalytic Properties of the Purified Ribulose bisphosphate carboxylase-oxygenase from Rhodopseudomonas blastica

4.3.1 Effect of pH on enzyme activity

The effect of pH on the catalytic activity of the Form I and Form II enzymes from R. blastica was studied with Tris-HCl buffer at different pH values. The enzymes were incubated in buffers ranging from pH 7.0 to 8.8 in the presence of Mg^{2+} (8.0 mM) prior to initiation of the reaction with RuBP. Figure 4.10 shows the effect of pH on the RuBP dependent $^{14}CO_2$ fixation by the Form I and Form II enzymes. The Form I enzyme had a broad peak of activity ranging from pH 7.2 to 8.2, with a maximum at pH 8.0. More than 50% of the maximal activity of the Form I enzyme was retained at pH 8.6. This observation is consistent with those for enzyme from plant sources (Kawashima and Wildman, 1970), the Form I enzyme from R. sphaeroides (Gibson and Tabita, 1977a) and Rhodomicrobium vannielii (Taylor, 1979).

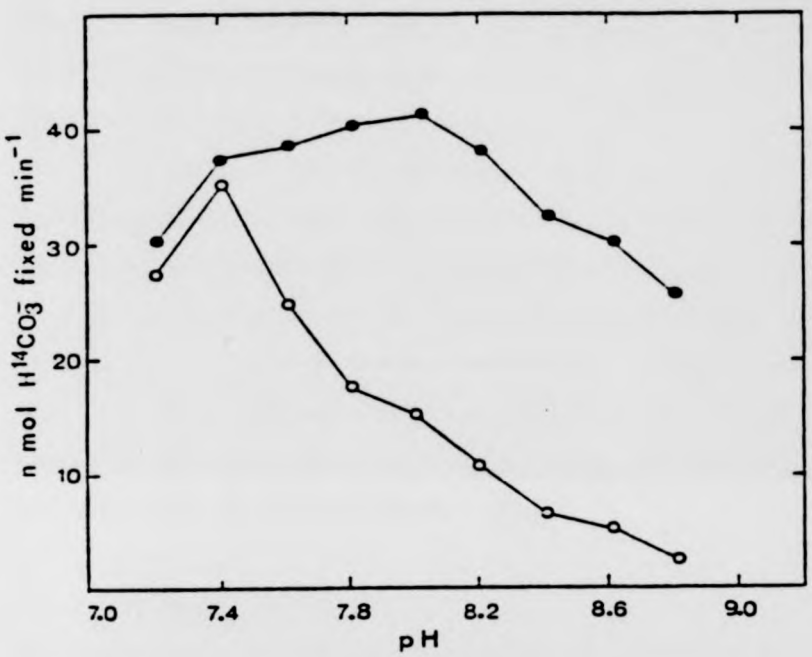


Figure 4.10 The effect of pH on the activity of Form I and Form II RuBisCOs from Rhodopseudomonas blastica

The standard reaction mixture contained Tris-HCl at the desired pH. The Mg^{2+} concentration in this experiment was 8 mM. (●) Form I; (○) Form II.

The purified Form II enzyme had maximal activity at pH 7.4, which decreased sharply to about 30% of its maximum at pH 8.0. At pH 8.6, the Form II enzyme retained only 15% of its maximal activity. The means by which the drastic effect of pH on the activity of the Form II enzyme is brought about is not understood. However, it may be as a result of enzyme denaturation or modification of structure or a combination of both. Certainly, a more sophisticated approach, e.g. using X-ray crystallography to study the enzyme structure during activation and deactivation, is required to elucidate this question. The differences in response to pH between the Form I and the Form II enzyme may well be a consequence of the small subunit functioning to protect or stabilize the Form I enzyme, although there is no direct evidence to support this view. The Form II enzyme from R. sphaeroides also exhibits a similar response to pH (Gibson and Tabita, 1977a).

4.3.2 Oxygenase Activity

The ability of all ribulose bisphosphate carboxylases thus far examined to function also as oxygenases, has been thought to be a consequence of the active site chemistry of the enzyme (Lorimer, 1981b). The oxygenase activity of the two forms of Ribulose bisphosphate carboxylase purified from Rhodopseudomonas blastica was therefore investigated using fractions from the DEAE Trisacryl columns and purified enzymes (Table 4.2). In the absence of Ribulose bisphosphate (RuBP) oxygen uptake was negligible for both enzymes. Addition of RuBP to the reaction mixture stimulated oxygen uptake. The specific activities of the oxygenase reaction catalysed by the purified Form I and Form II enzymes were 0.13 and 0.19 μmol oxygen consumed.min^{-1} mg $protein^{-1}$ respectively. Although the Km for O_2 was not determined, it is possible that the two enzymes have different affinities for O_2 as has been demonstrated for purified Form I and Form II RuBisCO

Table 4.2 The oxygenase activity of ribulose bisphosphate carboxylase from Rhodopseudomonas blastica

Enzyme Source Test substrate	Specific Activity [μmol of O_2 consumed. min^{-1} $(mg\ protein)^{-1}$]	
	Form I	Form II
DEAE-Trisacryl eluates	0.001	0.002
DEAE-Trisacryl elutates + RuBP	0.07	0.09
Sucrose gradient eluates + RuBP	0.13	0.19
Boiled enzyme	0	0

enzymes from R. sphaeroides (Jordan and Ogren, 1981). Nevertheless, the RuBP dependent oxygenase activities of the Form I and Form II enzymes from R. blastica are consistent with the enzymes from eukaryotic and prokaryotic sources thus far examined (Table 1.4, Chapter 1). Whether or not the oxygenase activity of the enzymes from R. blastica, and indeed from most photosynthetic bacteria, is of any in vivo physiological significance is not known, since these organisms are normally cultured under anaerobic conditions and do not evolve oxygen during photosynthesis.

4.3.3 Cation Requirement

The requirement of the purified enzymes from R. blastica for divalent cations for catalytic activity was investigated with enzyme dialysed against 20 mM Tris-HCl buffer (pH 8.0 for Form I and pH 7.4 for Form II enzyme) containing 5 mM EDTA. The Form I enzyme was assayed at pH 8.0 and the Form II at pH 7.4. Table 4.3 shows the effect of the different cations on the catalytic activity of the purified enzymes. The two enzymes did not incorporate $^{14}CO_2$ when assayed in the absence of Mg^{2+}. Pre-incubation of the enyzmes with assay mixtures containing 10 mM Mg^{2+} and $H^{14}CO_3$ and initiation of reaction with RuBP restored the maximal catalytic activity for both forms of the enzyme. Furthermore, at saturation levels of RuBP and $NaHCO_3$, normal Michaelis-Menten kinetic data were obtained for both enzymes at several concentrations of Mg^{2+} (Figure 4.11). However, the catalytic activity of the purified Form I enzyme was partially restored when assayed in the presence of Co^{2+} and Ca^{2+} (Table 4.3). The substitution of Mg^{2+} by Co^{2+} is in agreement with data reported for enzyme from plant sources (Kawashima and Wildman, 1970) and a number of T- type RuBisCOs from bacterial sources (Taylor and Dow, 1980; Tabita, 1981). However, the purified Form II RuBisCO from

Table 4.3 Effect of Divalent cation on the carboxylase activity of Form I and Form II ribulose bisphosphate carboxylase/ oxygenase from Rhodopseudomonas blastica

Cation (final concentration - 10 mM)	Percentage activity with Mg^{2+}	
	Form I	Form II
None	0.08	0.06
Mg^{2+}	100	100
Al^{2+}	2.5	1.3
Ca^{2+}	10.5	1.1
Cu^{2+}	2.2	1.0
Mn^{2+}	1.4	1.9
Ni^{2+}	2.2	0.8
Co^{2+}	12.2	1.2

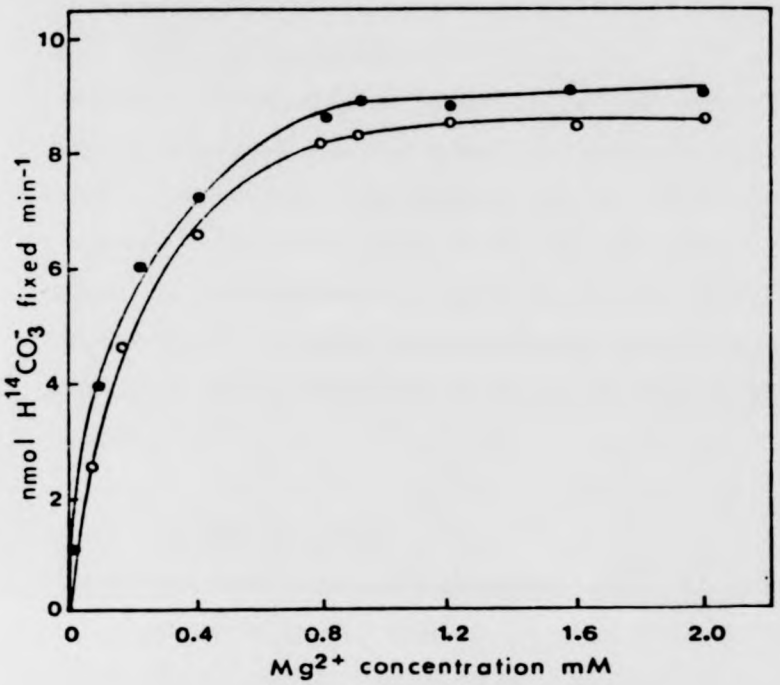


Figure 4.11 Effect of Mg^{2+} concentration on the carboxylase activity of Form I (●) and Form II (○) RuBisCO from Rhodopseudomonas blastica

R. blastica appeared to be specific for Mg^{2+}, since Al^{2+}, Ca^{2+}, Cu^{2+}, Ni^{2+}, Co^{2+} at a concentration of 10 mM and Mn^{2+} at 5 mM did not restore activity. The observation is similar to that reported for the O-type RuBisCO from Rhodospirillum rubrum (Tabita and McFadden, 1974a) and the Form II enzyme from R. sphaeroides (Tabita, 1981). This property therefore, seems to be common to the RuBisCO enzymes that lack the small subunit. The differences in the response of the Form I and the Form II RuBisCO from R. blastica to divalent cation reactivation may be a reflection of the differences in the active site chemistry of these two enzymes.

4.3.4 Kinetic Constants

Initial velocity studies and subsequent double reciprocal plots were used to determine the kinetic constants, i.e. Km for Ribulose bisphosphate (RuBP) and Km for 'CO_2' of the Form I and the Form II enzymes from Rhodopseudomonas blastica. The Form I and Form II enzyme had Kms for RuBP of 48 μM and 51 μM respectively, when determined at concentration of RuBP ranging from 10 to 200 μM (Figure 4.12). In both cases, the enzyme reactions were carried out under standard assay conditions, i.e. the enzymes were pre-incubated with saturation levels of Mg^{2+} and $H^{14}CO_3$ and reactions were started by the addition of RuBP and exhibited normal Michaelis-Menten kinetics. It therefore appears that the Form I and Form II enzymes have the same affinity for RuBP. The Kms(RuBP) for the enzymes from R. blastica are in agreement with those reported for the RuBisCO enzymes from plants (Jensen and Bahr, 1977), R. rubrum (Tabita and McFadden, 1974b), Ectothiorhodospira halophila (Tabita and McFadden, 1976), Rhodomicrobium vannielii (Taylor, 1979) and some chemosynthetic bacteria (Purohit et al., 1976a), but differed from that from Alcaligenes eutrophus which has a Km (RuBP) of 590 μM (Purohit and

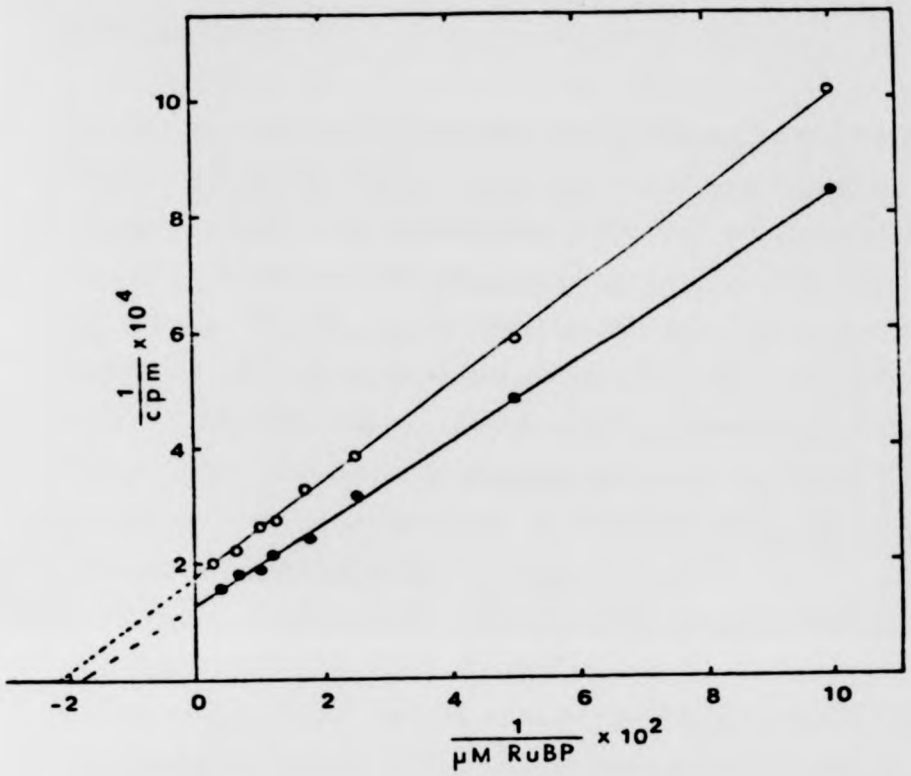


Figure 4.12 Effect of RuBP concentration on R. blastica ribulose bisphosphate carboxylase activity

The Lineweaver-Burk plot for the determination of Km (RuBP). (●) Form I; (○) Form II.

McFadden, 1977).

In the past, the kinetic constants for RuBisCO, especially Km (CO_2), have been considered too high to account for the *in vivo* activity of the enzyme (Kawashima and Wildman, 1970). This has been attributed to failure to attain full activation during the assay procedures (Lorimer *et al.*, 1976). Furthermore, the order of addition of co-factors and substrates is reported to affect the rate of reaction (Chu and Bassham, 1973; Tabita and McFadden, 1974b). In the determination of Km (CO_2) for the purified enzymes from *R. blastica* therefore, enzymes were fully activated in the presence of Mg^{2+} as described by Yeoh *et al.* (1980) and the reaction started with RuBP.

The double reciprocal plots of specific activity against increasing concentration of CO_2, when provided as $NaH^{14}CO_3$, gave Km 'CO_2' of 40 μM and 102 μM for the Form I and Form II enzymes respectively, at pH 7.4. This implies a marked difference in the affinity of the enzymes for CO_2, with Form I having a higher affinity for CO_2 than the Form II enzyme from the same organism. The Km 'CO_2' for the Form I enzyme is in general, within the range (9 to 56 μM) reported for most plant and algae sources (Yeoh *et al.*, 1980; Jordan and Ogren, 1981), and close to the value of 36 μM reported for the Form I enzyme from *R. sphaeroides* (Jordan and Ogren, 1981). As has been found with many other RuBisCOs investigated, this value may account for the *in vivo* activity of the enzyme (Badger and Andrew, 1974). On the other hand, the Km 'CO_2' of the Form II enzyme from *R. blastica* is similar to that reported for the O-type enzymes from *R. rubrum* and the Form II of *R. sphaeroides* (89 and 80 μM respectively) (Jordan and Ogren, 1981). Christeller and Laing (1978) showed that Km 'CO_2' for whole cells of *R. rubrum* decreases with increasing pH when

determined using 'CO_2' as the substrate, but there was no correlation between pH and Km (CO_2) when HCO_3 was used. Since the Form I and Form II enzymes from R. blastica exhibit different pH optima and also respond differently to changes in pH of the assay mixture, (Section 4.3.1 of this thesis), the Km 'CO_2' at varying pH was not determined. Nevertheless, the Form I enzyme has kinetic constants similar to the T-type enzymes from plants, algae and the Form I of R. sphaeroides, while that of the Form II enzyme is similar to the O-type RuBisCOs from R. rubrum and R. sphaeroides Form II. These correlations suggest that high CO_2 specificity requires the presence of both large and small subunits.

4.3.5 Inhibition by 6-Phosphogluconate

The effect of 6-phosphogluconate (6-PGluA) on the activity of the purified Form I and Form II ribulose bisphosphate carboxylase/oxygenase from Rhodopseudomonas blastica was investigated (Figure 4.13). When included in the incubation mixture, before initiation of the reaction with RuBP, 6-PGluA was found to be an effective inhibitor of the Form I enzyme. The sensitivty of the Form I enzyme was found to be dependent on the concentration of the inhibitor, 6-PGluA. The Form I enzyme showed a 50% inhibition at a concentration of 1.0 mM 6-PGluA and more than 80% at 2.0 mM. Furthermore, over a concentration range of 0.2 to 0.6 mM, 6-PGluA showed a linear competitive inhibition with RuBP.

In contrast to these observations, the Form II enzyme was relatively insensitive to inhibition by 6-PGluA (Figure 4.13). In fact, at concentrations between 0.2 and 0.8 mM, 6-PGluA was a positive effector of the Form II enzyme, with its activity increasing to 107% of that observed in the absence of 6-PGluA. The Form II enzyme showed only a slight inhibition (10%) with 2.0 mM 6-PGluA.

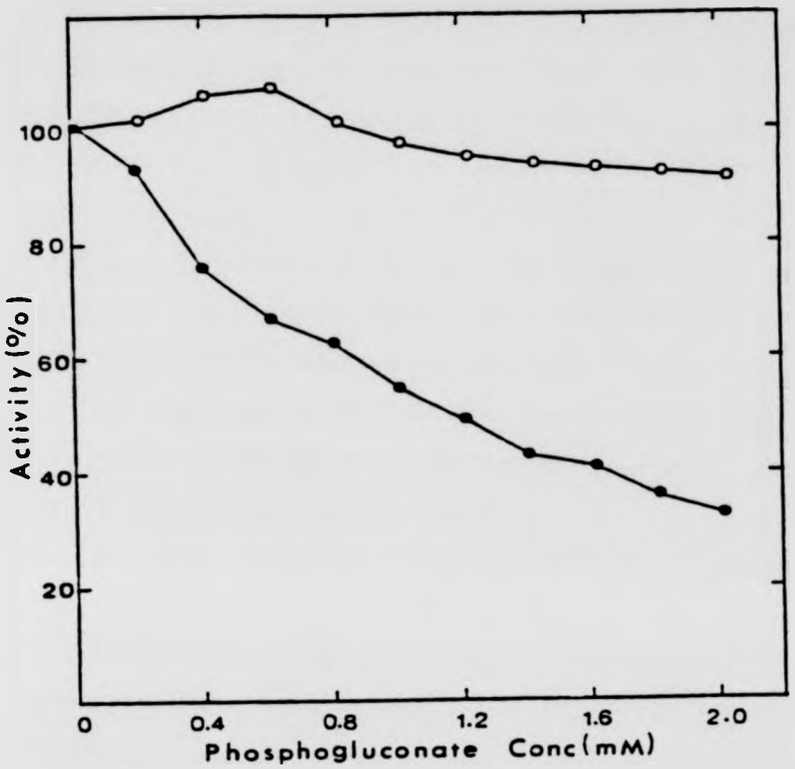


Figure 4.13 The effect of 6-phosphogluconate on the activity of Form I (●) and Form II (○) RuBisCO from Rhodopseudomonas blastica

Enzymes were preincubated for 10 min at 30°C in the presence of Mg^{2+}, $H^{14}CO_3$ and 6-phosphogluconate in Tris-HCl buffer prior to initiation of the reaction with RuBP. Form I enzyme was assayed at pH 8.0, and Form II at pH 7.4.

The inhibition of the Form I enzyme by 6-PGluA is similar to that reported for the large molecular size and T-type enzymes from plant and a number of bacterial sources (Kawashima and Wildman, 1970; Tabita and McFadden, 1972, 1976; Bowman and Chollet, 1980; Tabita, 1981; Taylor and Dow, 1980). The insensitivity of the Form II enzyme from R. blastica to 6-PGluA is consistent with the observations made with O-type enzymes from R. rubrum, and the Form II RuBisCOs from R. sphaeroides and R. capsulata (Tabita and McFadden, 1974b; Gibson and Tabita, 1977a, b). The findings that 6-PGluA inhibits only the Form I but not the Form II RuBisCO from R. blastica may indicate that the two enzymes have marked differences in the topography of the active site which may be a reflection of their quaternary structures.

4.4 Peptide Mapping of the large subunits of Form I and Form II ribulose bisphosphate carboxylase/oxygenase from Rhodopseudomonas blastica

One of the most useful methods for the separation of polypeptide chains in complex biological samples is by electrophoresis in polyacrylamide gels. However, the identification of the relationships between specific proteins cannot be made on the basis of electrophoretic mobility alone. This may be true, for example, if closely related proteins differ in mobility due to slight chemical modification or as a result of artifactual proteolysis during the purification procedures. Many different techniques may be applied to this problem, e.g. the analysis of the amino acid sequence. However, this requires lengthy procedures and complete amino acid sequencing may not be feasible. Other methods that may be used to identify relationships between specific proteins include

immunological characterization and peptide mapping. The use of immunological characterization may not be conclusive evidence for non-relatedness of two polypeptides, since non-cross reactivity between one polypeptide and the antibody raised against the other may well be a consequence of slight modification(s) of the antigenic determinant of the polypeptide. Peptide mapping or "finger printing" has been considered as a stringent test of protein identity (James, 1980). This method is specific and also allows for digests of different polypeptides to be compared under identical experimental conditions.

The Form I and Form II RuBisCO purified from R. blastica are different in terms of molecular and kinetic properties. Whether or not the differences are due to the presence of the small subunit in the Form I enzyme cannot be concluded from the kinetic properties alone. Furthermore, it cannot be assumed from the differences in the kinetic and molecular properties that the two enzymes are products of different genes. The relatedness of the Form I and Form II RuBisCOs from R. blastica was therefore, investigated by peptide mapping of the two large subunits. Tryptic digests of the isolated large subunits were compared by analysis of the products of the digests by SDS polyacrylamide gel electrophoresis.

In this study, the method of limited tryptic digestion was used to compare and contrast the substructure of the large subunits of the Form I and Form II RuBisCO purified from R. blastica. Trypsin, a protease that is specific for cleavage of peptide bonds in which the carboxyl group is contributed by lysine and arginine, was used to digest the isolated large subunits. The banding pattern of the peptide fragments generated by the tryptic digestion are shown in Figure 4.14. At a fixed protease

concentration it can be seen that with increasing time of digestion, there is a progressive disappearance of the large subunit bands of the Form I and the Form II enzymes. However, it is notable that the two paterns are completely distinct, with each digest containing many peptide fragments of mobility not present in the other. The large subunit of the Form I enzyme generated up to 10 distinct peptide fragments after incubation with trypsin for 60 min, while the Form II enzyme had about 21 fragments, under the same conditions of digestion. The apparent molecular weights of the peptide fragments generated from the large subunit of the Form I enzyme ranged from 11,000 to 49,000 while those for the Form II enzyme range from 10,000 to 45,000 (Figure 4.14). Of the total peptide fragments generated between 30 and 60 min of digestion with trypsin, five (a, 37; b, 20; c, 14; d, 13 and e, 12 K Dal) appeared to be common to the two enzymes.

The digestion of the large subunits of the Form I and Form II enzyme using increasing concentrations of trypsin, but at a fixed incubation time of 60 min, is shown in Figure 4.15. It can also be seen that the same pattern of peptide fragments which were present in the time course experiment (Figure 4.14) are again observed in this experiment. Similar to the time course experiments, fragments 37, 14, 13 and 12 K Dal are common to both large subunits and appeared to be stable to digestion by the protease. These fragments, except the 22 K Dal, may be the conserved region of the large subunit polypeptide chain of the Form I and Form II RuBisCOs from R. blastica. Fragment 22 K Dal may be that of the protease since it co-migrated with the trypsin band (T) (Figure 4.15).

The marked insensitivity of the digestion to large variations in protease concentration and in the length of incubation with protease indicates

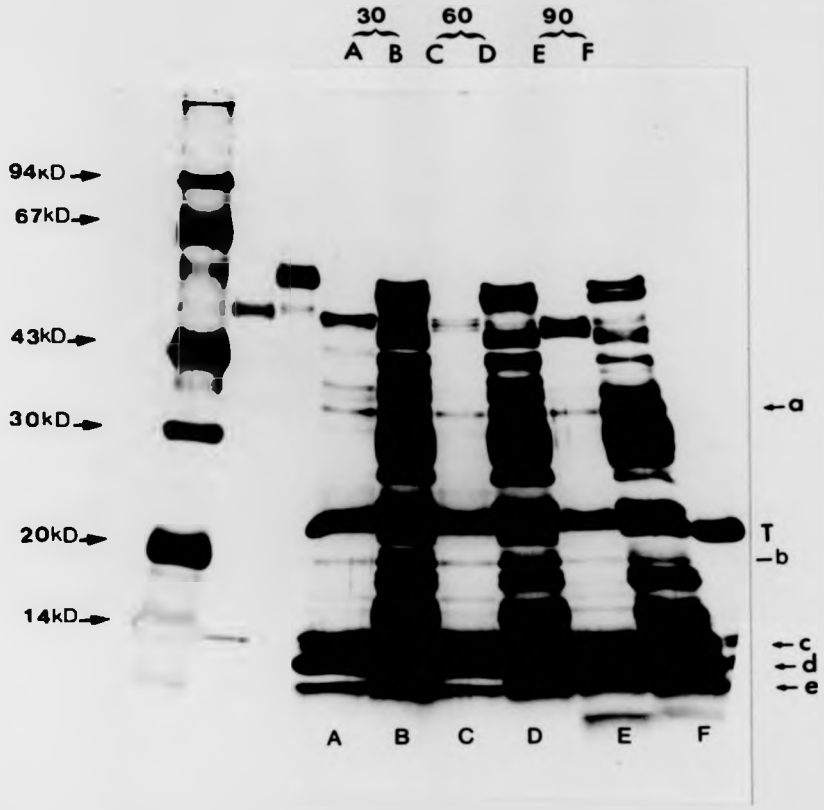
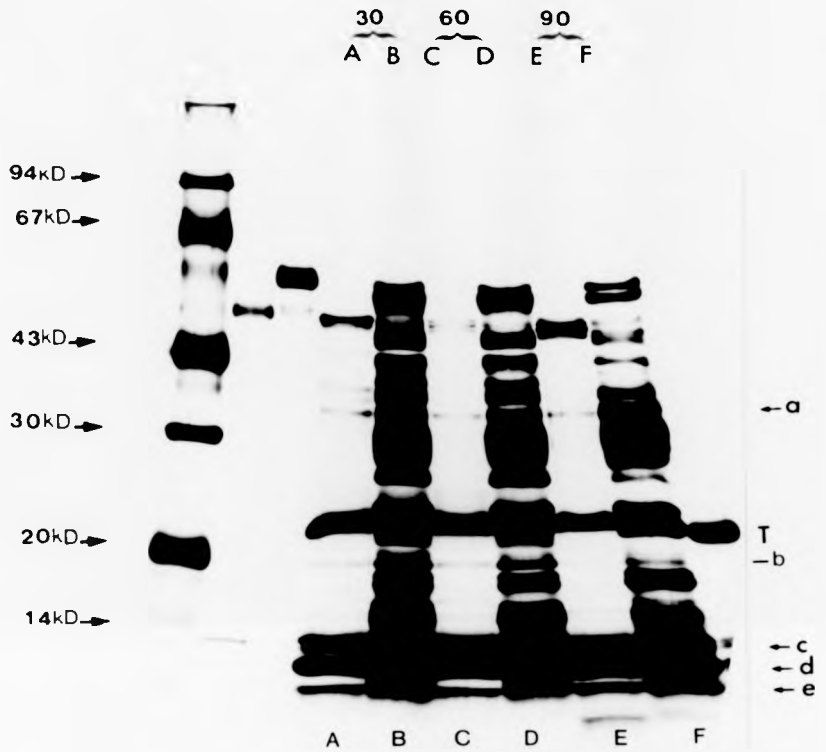


Figure 4.14 Peptide mapping of the large subunits of the Form I and Form II RuBisCO from Rhodopseudomonas blastica: Kinetic study of digestion with trypsin

Form I large subunit (A, C and E) and Form II (B, D and F) were digested at 37°C with trypsin at a final concentration of 25 µg/ml (labelled T). The length of incubation was varied between 30 and 90 mins as indicated in the figure.



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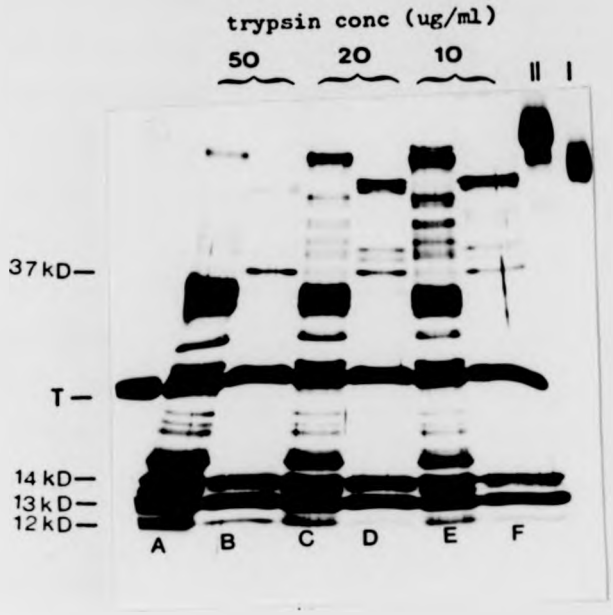


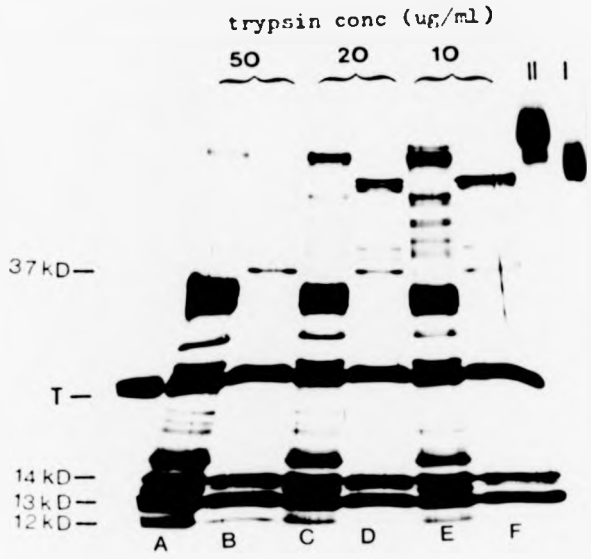
Figure 4.15 Effect of increasing concentration of trypsin (µg/ml) on the digestion of isolated large subunits of Form I and Form II RuBisCOs from R. blastica

The large subunits of the Form I (A,C,&E) and Form II (B,D,F) were digested at 37°C with trypsin.

In all cases enzyme was incubated with trypsin for 60 min.

I=Large subunit of Form I

II = Large subunit of the Form II enzyme



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The large subunits of the Form I (A,C,&E) and Form II (B,D,F) were digested at 37°C with trypsin.

In all cases enzyme was incubated with trypsin for 60 min.

I=Large subunit of Form I

II = Large subunit of the Form II enzyme

that the patterns of the peptide fragments is reproducible. The differences in the pattern of fragments generated from the two large subunits of RuBisCO enzymes which are independent of length of incubation with protease or the concentation of protease used, suggests that the two forms of RuBisCO from R. blastica are different enzymes. They may indeed, be products of different genes. Certainly, further substantiation of these results will require the knowledge of the amino acid composition and the sequence of the two large subunits of the RuBisCO enzymes. In addition, the genes coding for the two enzymes could be cloned, the percentage hybridization determined and ultimately the DNA sequence.

Conclusion

Two major and distinct molecular forms of ribulose-bisphosphate carboxylase/oxygenase have been isolated from extracts of R. blastica. One of these enzymes structurally resembles the T-type RuBisCO from plant sources while the other is of the O-type, lacking the small subunit. The kinetic properties and peptide "finger printing" of the isolated large subunits of the two enzymes show that the Form I and Form II RuBisCOs are quite unrelated and are possibly products of different genes. However, there is the need to further substantiate this conclusion by, e.g. determining the amino acid sequence of the two large subunits, or by cloning and DNA sequencing. An understanding of the physiological significance of the presence of two different molecular forms of RuBisCO in R. blastica may provide an insight into how CO_2 metabolism is regulated in the photosynthetic prokaryotes.

Chapter 5

Results and Discussion III

Physiology of the Synthesis of Ribulose-1,5-bisphosphate Carboxylase/Oxygenase in *Rhodopseudomonas blastica*

5.1 Introduction

Many bacteria are able to adjust the level and expression of enzymes involved in various metabolic pathways in response to variations in medium composition and growth conditions. This is obviously of advantage to the cells since enzymes are synthesised to meet specific requirements. In general, enzyme synthesis can be regulated at different levels: a) at the transcriptional level - a mode of control which consequently influences the rate of synthesis of specific enzymes. In this respect, two mechanisms can be recognised, namely, regulation by induction (as with some inducible enzymes, e.g. β-galactosidase) or by repression of enzyme synthesis which can be catabolic or end-product repression (Watson, 1975).

b) Enzyme synthesis can also be regulated at the translational level (Lodish, 1976), and this can be achieved by either controlling the stability of a particular mRNA or the rate of initiation of mRNA translation. Other modes of enzyme regulation include feedback or end-product inhibition and catabolic inhibition. In either case, the effects are directed at synthesised enzymes.

Most of the knowledge concerning regulatory mechanisms of enzyme synthesis has been obtained by growing microorganisms in batch cultures at high substrate concentrations. It might be expected however, that in nature, most substrates are present at sub-saturation levels. One might wonder therefore, whether the regulatory mechanisms of enzyme synthesis observed at high substrate concentration also play a significant role in the natural environment. This can be investigated by studying enzyme regulation under substrate-limiting conditions. These conditions can be best achieved using continuous culture techniques, where organisms can be

studied at different concentrations of growth limiting substrates, i.e. at different dilution rates (D) (Herbert et al., 1956).

By monitoring the changes that occur in cell physiology at different dilution rates regulatory mechanisms can often be identified. Four different responses in enzyme activity in relation to dilution rate can be recognised (Dean, 1972; Matin et al., 1976).

1. Enzyme specific activity remains constant irrespective of dilution rate. This type of response is thought to occur in the case of constitutive enzymes (Dijkhuizen, 1979).

2. Enzyme specific activity increases with increase in dilution rate. This is generally found for enzymes which are induced by growth-limiting substrate since in this case an increase in the inducer concentration will be expected with an increase in dilution rate.

3. Specific activity increases as the dilution rate is decreased. This is the case when the synthesis of an enzyme is controlled by repression exerted by the growth limiting substrate. In this situation decreasing the dilution rate can cause a release of repression, since the repressor concentration will decrease with decreasing concentration of the growth limiting substrate (i.e. derepression of enzyme synthesis). This mode of control is exhibited by Pseudomonas sp. grown in continuous culture under steady state conditions in L-lactate, succinate, ammonium or phosphate limited media (Matin et al., 1976).

4. In a system whereby enzyme synthesis is controlled by both induction and repression exerted by the growth-limiting substrate, a more complex

situation is obtained as shown in Pseudomonas aeruginosa grown on acetamide (Clarke et al., 1968). It was found that in continuous culture, the specific activity of the inducable amidase of P. aeruginosa was determined by the balance between induction and catabolic repression.

It is clear from this brief survey that the application of continuous culture techniques to the study of enzyme regulation is more sophisticated than batch culture methods. In continuous culture, enzyme regulation can be studied under steady state conditions over a range of dilution rates and thus over a range of concentrations of the growth-limiting substrate. In this way the physiology of enzyme synthesis, and of the organism in general, can be studied under conditions which closely resemble those in the natural environment where low and limiting concentrations of carbon and energy sources is the predominant condition.

Up to now, the limited information on the physiology of synthesis of ribulose bisphosphate carboxylase/oxygenase and the regulation of CO_2 fixation in the Rhodospirillaceae has been obtained with organisms grown in batch cultures. It was therefore of interest to investigate these control mechanisms using continuous culture techniques. Rhodopseudomonas blastica, like Rhodopseudomonas sphaeroides, synthesises two different molecular forms of Ribulose bisphosphate carboxylase-oxygenase (Section 4.1 of this thesis; Gibson and Tabita, 1977a). In the latter organism, it is not known how the synthesis of the two forms of the RuBisCO enzymes is regulated. The ability of Rhodopseudomonas blastica to grown under both aerobic and anaerobic conditions was therefore, exploited in continuous culture to study the effect of cultural conditions on the physiology of synthesis of RuBisCO and the regulation of CO_2 fixation.

In this study, R. blastica was grown to steady state in continuous culture under different limitations, and CO_2 fixation in soluble protein extracts and whole cells determined. The soluble protein extracts were then subjected to partial purification by sucrose gradient centrifugation and subsequently analysed for the presence of the two forms of the RuBisCO enzyme by the non-denaturing polyacrylamide gel assay system previously described (Section 2.18).

5.2 The Effect of Carbon Source on the level of Ribulose Bisphosphate Carboxylase/Oxygenase in cell-free Extracts of Rhodopseudomonas blastica

The key to obtaining homogeneous ribulose bisphosphate carboxylase-oxygenase (RuBisCO) from Rhodospirillum rubrum (Tabita and McFadden, 1974a, b) and Rhodopseudomonas sphaeroides (Gibson and Tabita, 1977a) was provided by photoheterotrophic growth with butyrate as the electon donor. This resulted in a significant expression of enzyme activity far more than that obtained with malate, the "usual" growth substrate for the Rhodospirillaceae. This effect was investigated in R. blastica by measuring RuBisCO activity in cell free extracts after growth on a range of carbon substrates. For comparative purposes the enzyme activity was determined in soluble protein extracts of cells from exponential and early stationary phases of growth. This was with a view to investigating the effect of high (saturation level) and low (limiting) substrate concentrations on the expression of RuBisCO in this organism during batch growth. Table 5.1 shows the effect of different carbon substrates on the specific activity of RuBisCO in soluble extracts of R. blastica. Clearly, the specific activities of this enzyme are low (repressed) under

Table 5.1 Growth-substrate-dependent synthesis of ribulose bisphosphate carboxyase/oxygenase in Rhodopseudomonas blastica

Substrate	Enzyme activity (μmol $^{14}CO_2$ fixed.min^{-1}.mg protein^{-1})	
	mid-exponential	early stationary
Acetate	0.008	0.01
Acetate + CO_2	0.0092	0.0112
Glucose	0.019	0.023
Pyruvate	0.010	0.014
Pyruvate + CO_2	0.011	0.016
Malate	0.018	0.021
Pyruvate-malate	0.033	0.040
Succinate	0.014	0.021
Succinate + CO_2	0.015	0.019
Butyrate + CO_2	0.015	0.14

all conditions of photoheterotrophic culture when cells are harvested from the exponential phase of growth. During this growth phase, the highest specific activity (0.033 U $.min^{-1}.mg\ protein^{-1}$) was obtained from the soluble extract of cells grown in pyruvate-malate medium. This value is similar to that obtained for R. sphaeroides grown on malate (Tabita, 1981). Interestingly, cells harvested from the early stationary phase of growth on butyrate-bicarbonate medium showed a 9-fold increase in the RuBisCO specific activity compared with that from the exponential phase of growth on the same medium (Table 5.1). This effect was not observed for other carbon substrates (pyruvate, malate, glucose, acetate and succinate) tested (Table 5.1). Under these growth conditons, RuBisCO activities in the soluble extracts are found to be low (repressed) in the early stationary phase of growth. It is worth noting that addition of CO_2 supplied as bicarbonate to the growth media other than butyrate, did not have any effect on the level of RuBisCO in the soluble extracts of R. blastica. The high level of RuBisCO in the soluble extract of R. blastica from the early stationary phase of growth on butyrate-HCO_3 medium, may indicate that the signal for the induction of the enzyme is the cells becoming limiting for the substrate - presumably CO_2. Similar results were obtained with the physiologically related organisms, R. sphaeroides (Gibson and Tabita, 1977a) and R. rubrum (Tabita and McFadden, 1974a, b; Tabita, 1981).

The low levels of RuBisCO in cells grown on either pyruvate, malate, glucose, acetate or succinate may be due to the nature of these organic carbon substrates rather than their concentrations. This will be so, since the RuBisCO activities from cells in the exponential phase (when substrate is present at high levels) are similar to those from the early stationary phase (when the substrate will become limiting). The control

of expression of RuBisCO in R. blastica may well be under the influence of other factors such as light, oxygen and growth rates. The influence of these factors, in addition to CO_2 limitation, on the control of synthesis of RuBisCO in R. blastica was therefore studied in chemostat cultures.

5.3 The Effect of Cultural Conditions on the Level of Ribulose bisphosphate carboxylase/oxygenase in Rhodopseudomonas blastica grown in continuous culture

Carbon dioxide fixation by the soluble extract and whole cells of R. blastica was examined in steady-state cultures grown under different limitations in the chemostat (Table 5.2). Under aerobic growth conditions in the light (malate as carbon source), CO_2 fixation by the soluble extract was low (2.2 nmol.min^{-1}.mg $protein^{-1}$ and this was parallelled by a low level of CO_2 fixing activity by the whole cells. Similarly, under dark aerobic growth conditions, low levels of RuBisCO activity were observed in the soluble protein extracts. This suggests that light per se had no effect on the induction of this enzyme under aerobic growth conditions since enzyme levels in cells grown either in the dark (chemoheterotrophically) or in the light on malate were similar (Table 5.2).

Under anaerobic growth on malate at high (10,000 lux) and low (2,000 lux) light intensities the CO_2 fixing activity by the soluble extracts increased 4 and 5-fold respectively over that for aerobic growth in the light. However, under anaerobic growth conditions, it seemed that the carbon source, i.e. malate, was more directly involved with the control

Table 5.2 Growth of Rhodopseudomonas blastica in continuous culture under different cultural conditions

Growth conditions ($D = 0.05\ h^{-1}$)	Protein $mg.ml^{-1}$	Enzyme activity (nmol $^{14}CO_2$ fixed $min^{-1}.mg\ protein^{-1}$)	$^{14}CO_2$ fixation by whole cells (nmol $H^{14}CO_3^{-1}$ fixed.mg dry weight$^{-1}.h^{-1}$)	Dry weight ($mg\ l^{-1}$)
Light anaerobic (malate limited)	5.50	9.8	43.3	408
Light aerobic (malate limited)	7.0	2.2	4.2	376
Dark aerobic (malate limited)	6.0	2.7	0.0	378
Light anaerobic (CO_2 limited)	4.7	12.7	134.0	380
Light anaerobic CO_2 limited + butyrate as substrate	5.0	176.0	546.0	409

of synthesis of RuBisCO in R. blastica than was light, since enzyme activities at high and limiting light intensities were similar and were present at low (repressed)levels. Interestingly, under CO_2 limited growth with butyrate, enzyme activity in the soluble extracts increased by 75 and 18-fold over that of aerobic and anaerobic (light intensity of 10,000 lux) growth respectively (Table 5.2). Similarly, the CO_2 fixing activity by the whole cells increased from 4.2 nmol.mg dry $wt^{-1}.h^{-1}$ under aerobic growth in the light to 546 nmol.mg dry $wt^{-1}.h^{-1}$ under CO_2 limited growth. The high specific activity of RuBisCO in the soluble extract of R. blastica under CO_2 limited growth, probably reflected high in vivo levels (derepressed) of this enzyme. It should be noted that under the different limitations in the chemostat, cells were grown at the same dilution rate (0.05 h^{-1}) and biomass production in terms of dry weight of cells, were of the same order of magnitude (Table 5.2), yet there was enormous differences in the RuBisCO activity (in the soluble extracts and whole cells) between CO_2 limited and malate grown cells. This may suggest that the high enzyme activity in the soluble extract of CO_2 limited cells was due to increasing synthesis of RuBisCO rather than activation of preformed enzyme.

5.4 Effect of Dilution Rate on the Activity of Ribulose Bisphosphate Carboxylase/Oxygenase in R. blastica during growth on malate

To determine whether the low levels of RuBisCO enzyme observed in cells grown on malate in the chemostat was growth rate dependent or substrate specific, R. blastica was grown at different dilution rates on malate mineral salts medium. Changing the dilution rate over a range of 0.001 to 0.14 h^{-1}, the culture biomass measured as absorbance or dry weight,

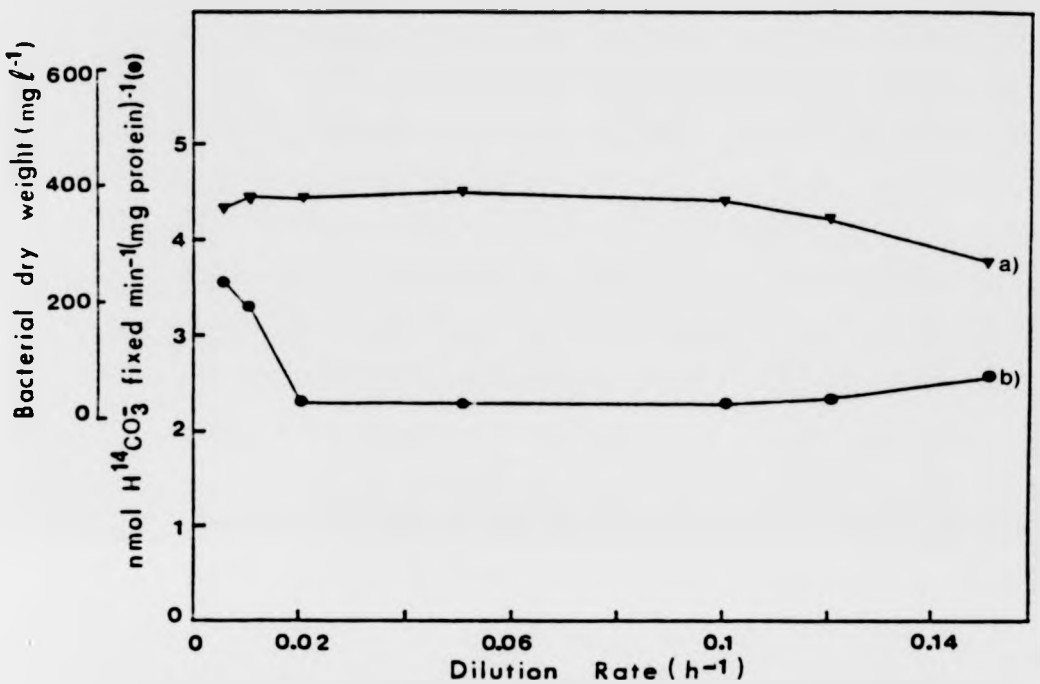


Figure 5.1 The effect of dilution rate (D) on (a) the yield and (b) the activity of RuBisCO from R. blastica grown in continuous culture

The mineral salts medium contained sodium hydrogen malate (6.6 mM final concentration) as carbon source.

showed the normal nutrient-limited chemostat pattern, with the dry weight remaining relatively constant at dilution rates up to 0.1 h^{-1}, thereafter, declining as the critical dilution rate was approached (Figure 5.1). The ribulose bisphosphate carboxylase specific activity (in terms of activity per unit protein) remained relatively constant at dilution rates between 0.02 and 0.12 h^{-1} [lowest activity was 2.3 nmol $^{14}CO_2.min^{-1}.(mg\ protein^{-1})$] and only increased slightly to 3.7 units as the dilution rate was decreased to 0.001 h^{-1}. It therefore appeared that, repression of RuBisCO synthesis in R. blastica grown on malate was due to the nature of this carbon substrate rather than the growth rate.

5.5 Concluding Remarks on the Nature of Regulation of the Synthesis of Ribulose Bisphosphate Carboxylase/oxygenasein Rhodopseudomonas blastica

It has been shown in this study that RuBisCO levels in R. blastica are a function of the mode of growth. In the presence of oxidizable organic substrates, e.g. malate, R. blastica synthesizes low repressed levels of RuBisCO independent of growth phase in batch culture. However, the growth of the organism photoheterotrophically on a reduced substrate such as butyrate-HCO_3, results in induction of high levels of RuBisCO, such that the specific activity in the soluble extracts is comparable to autotrophic growth of R. rubrum on molecular hydrogen and CO_2 (Anderson and Fuller, 1967b,c). In R. blastica. the RuBisCO enzyme is synthesised to high levels on butyrate-HCO_3 medium only during the early stationary phase of growth presumably due to the cells becoming limiting for CO_2.

The growth of R. blastica in continuous culture under different

limitations shows that this organism is metabolically versatile, and its response to changes in the environmental growth conditions results in large variations in the levels of the autotrophic enzyme, i.e. RuBisCO and indeed CO_2 fixation by whole cells. Under aerobic conditions on malate, either in the light or in the dark, strong repression of the synthesis of RuBisCO was observed as evident from the low specific activity of RuBisCO in the soluble extracts and CO_2 fixation by the whole cells. Strong repression of the synthesis of RuBisCO was also observed during photoheterotrophic growth on malate under anaerobic conditions, though enzyme specific activities were 4 to5 fold higher than those obtained from aerobically grown cells. However, under anaerobic conditions, enzyme specific activity was not significantly influenced by the light intensity.

The growth of R. blastica under CO_2-limitation with butyrate results in the derepression of RuBisCO. Similar results have been reported in a chemosynthetic bacteria, Alcaligenes eutrophus when grown under autotrophic conditons ($H_2/CO_2/O_2$ atmosphere) in continuous culture (Friedrick, 1982). In this organism it was demonstrated that the levels of RuBisCO in the cells was strongly dependent on the growth limiting factor, with the highest specific activity observed only under CO_2 limitation.

In conclusion, it is apparent that the synthesis of RuBisCO (and perhaps other key enzymes of the Calvin cycle) in R. blastica is regulated by a repression and derepression mechanism. In this organism, there is no indication that either activation or inactivation of the preformed enzyme is an important mode of control, rather derepression of the synthesis of RuBisCO is signalled by carbon (CO_2) limitation in the growth medium.

Maximal repression on the other hand occurred in the presence of oxidizable organic compounds, e.g. malate, pyruvate, succinate and acetate. These substrates are possibly metabolised heterotrophically and therefore ensured the supply of carbon intermediate for central metabolism. This will be of advantage to the cells since energy consuming CO_2 fixation is avoided under these conditions. This mode of control (i.e. repression) exerted by the metabolism of "heterotrophic" substrate has been observed in some members of the Rhodospirillaceae (Tabita et al., 1983), in hydrogen bacteria, e.g. A. eutrophus (Friedrick et al., 1981; Leadbeater et al., 1982) and in methylotrophs, e.g. Pseudomonas oxalaticus (Dijkhuizen et al., 1978; Dijkhuizen and Harder, 1984). These findings may indicate that in these three groups of bacteria, synthesis of the Calvin cycle enzymes is regulated in a similar fashion. However, R. blastica synthesises two different molecular forms of ribulose bisphosphate carboxylase-oxygenase (Section 4.1 of this thesis), it was therefore of interest to investigate which of the two forms is synthesised under different limitations.

5.6 Effect of Growth Conditions on the Expression of the Form I and Form II Ribulose Bisphosphate Carboxylase/Oxygenase in Rhodopseudomonas blastica Grown in Continuous Culture

As previously shown, R. blastica synthesises two different molecular forms of RuBisCO. Furthermore, the levels of the enzyme in the cells is affected by cultural conditions as demonstrated in chemostat culture (see above). In order to find out which of the two forms of the RuBisCO enzyme was synthesised in R. blastica during growth in the chemostat under different limitations, soluble extracts of cells were subjected to

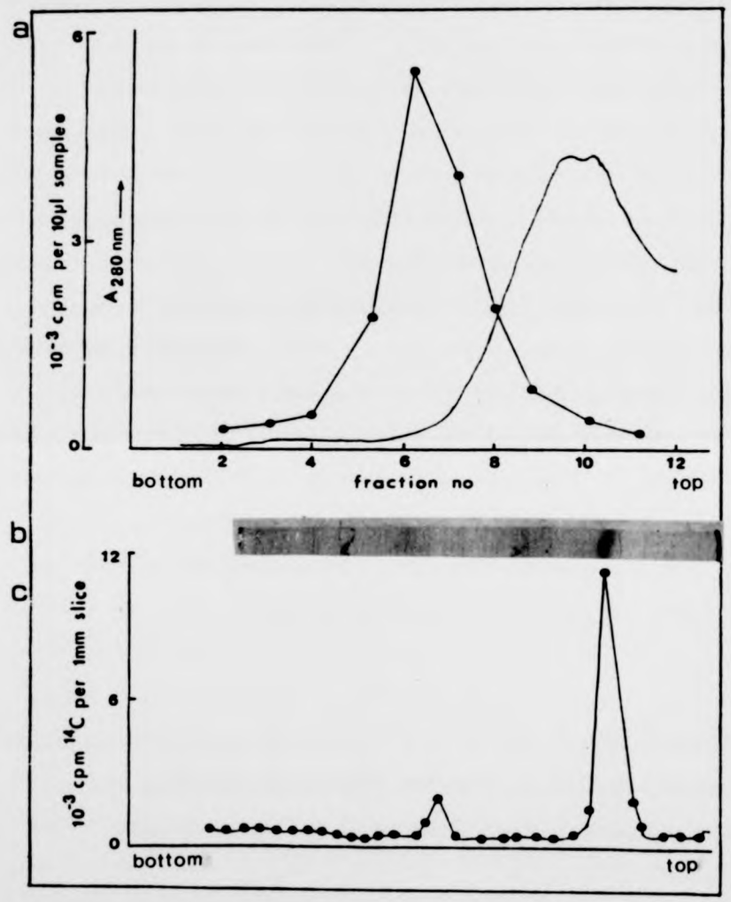
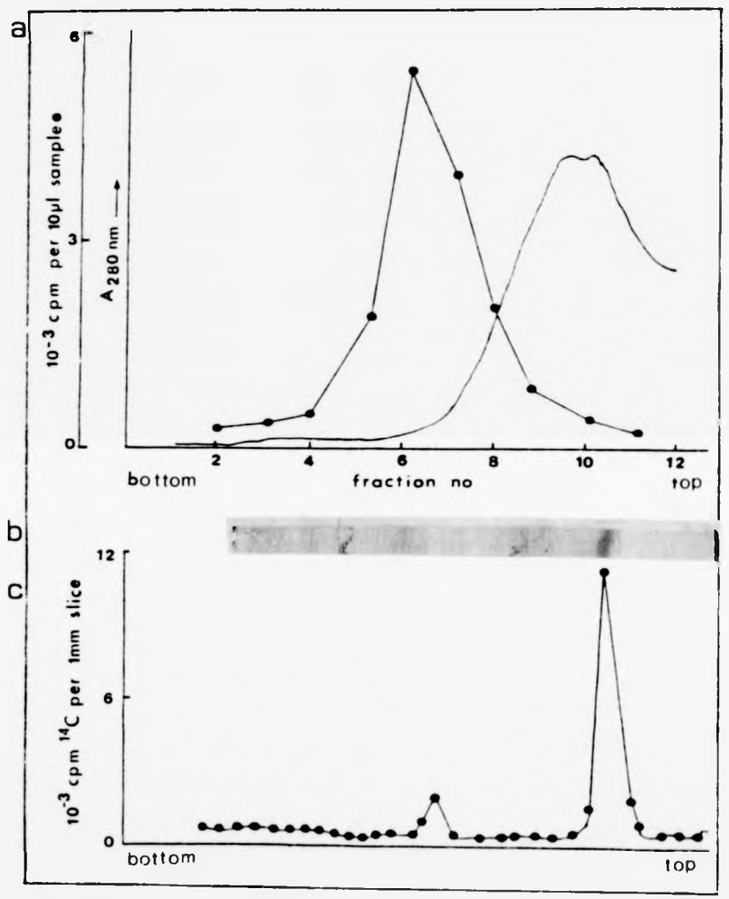


Figure 5.2 Soluble protein extract from anaerobic chemostat culture of R. blastica grown on malate mineral salts medium at 10,000 lux light intensity (D = 0.05 h^{-1}), subjected to sucrose gradient centrifugation procedure

a) A_{280} nm trace of the 0.2 to 0.8 M step sucrose gradient fractions superimposed on which is the RuBisCO activity (●).

b) Electrophoretogram of the 5% (w/v) non-denaturing polyacrylamide gel electrophoresis of pooled active fractions from (a).

c) RuBisCO activity in gel slices.



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b) Electrophoretogram of the 5% (w/v) non-denaturing polyacrylamide gel electrophoresis of pooled active fractions from (a).

c) RuBisCO activity in gel slices.

partial purification by the step sucrose gradient centrifugation procedure previously described (section 2.17). Pooled active RuBisCO fractions were then subjected to non-denaturing polyacrylamide gel electrophoresis and subsequently analysed by the in situ polyacrylamide gel assay for RuBisCO (as described under materials and methods). Figure 5.2a, shows the A_{280} of the soluble extract of photo-heterotrophically grown cells of R. blastica under anaerobic conditions at light intensity of 10,000 lux, upon which the ribulose bisphosphate (RuBP) dependent CO_2 fixing activity of the 1 ml fractions is superimposed. A sharp, symmetrical peak of CO_2 fixing activity separated from the bulk of the soluble protein is observed. Thus, active fractions were pooled and electrophoresed on non-denaturing polyacrylamide gels. Figure 5.2b shows the electrophoretogram of stained gel along with the RuBP carboxylase activity profile in the gel slices. One major peak of RuBP dependent CO_2 fixing activity, corresponding to the stained protein band in the gel was observed. Although the Form II enzyme was not detectable in the stained gel, a slight peak of CO_2 fixing activity in the gel slices was observed further down towards the bottom of the gel (Figure 5.2c). This might indicate that both the Form I and Form II enzymes are synthesised under this condition, though at low levels.

Partial purification and subsequent non-denaturing polyacrylamide gel analysis of the soluble protein extracts of R. blastica from light limited chemostat cultures, showed that the Form I and Form II enzymes were synthesised under such growth conditions (Figure 5.3). This is evident from the broad peak of RuBP dependent CO_2 activity in the sucrose gradient fractions (Figure 5.3a)and two peaks of CO_2 fixing activity in gel slices which correspond to two stained protein bands in the gel electrophoretogram of pooled fractions from the sucrose gradient

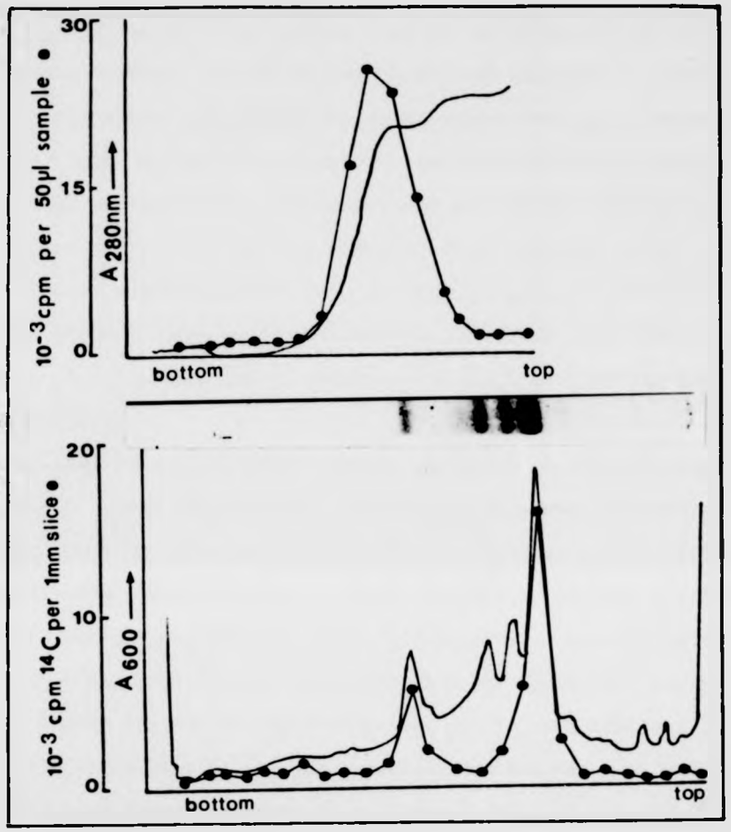

Figure 5.3 Soluble protein extract from a light limited chemostat culture (D = 0.05 h^{-1}) of R. blastica subjected to partial purification of the RuBisCO enzyme by sucrose gradient centrifugation procedure as described in Materials and Methods (Section 2.17)

a) A_{280} nm trace of the 0.2 to 0.8 M step sucrose gradient superimposed on which is the RuBisCO activity (●).

b) Active fractions from (a) were pooled and analysed by 5% (w/v) non-denaturing PAGE.

c) Densitometric scan of (b) and enzyme activity in gel slices.

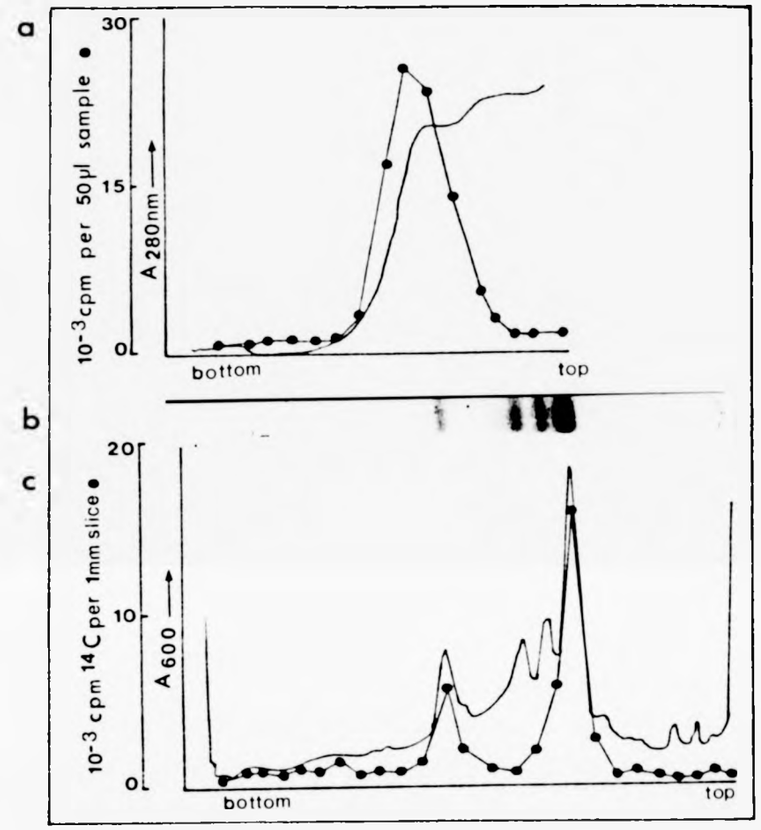

Figure 5.3 Soluble protein extract from a light limited chemostat culture (D = 0.05 h^{-1}) of R. blastica subjected to partial purification of the RuBisCO enzyme by sucrose gradient centrifugation procedure as described in Materials and Methods (Section 2.17)

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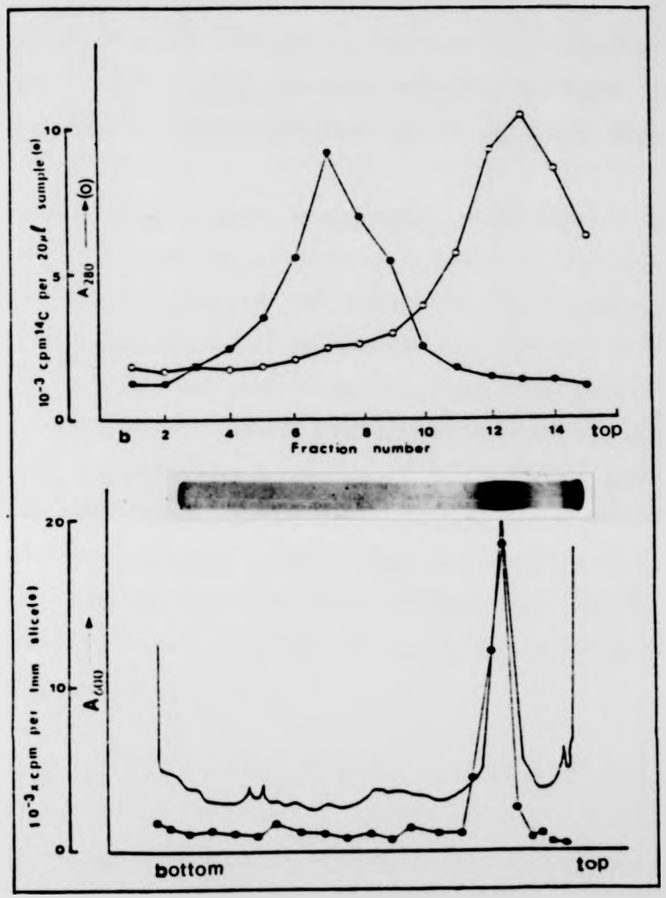


Figure 5.4 Soluble protein extract from CO_2 limited chemostat culture of R. blastica grown at a dilution rate of 0.05 h^{-1} subjected to analytical protocol as described for Figure 5.3.

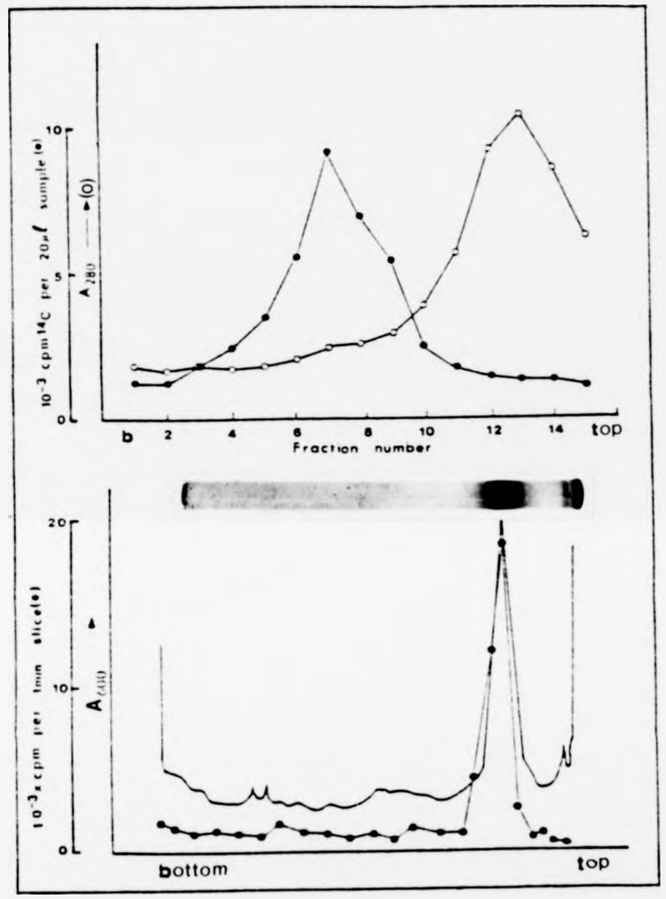


Figure 5.4 Soluble protein extract from CO_2 limited chemostat culture of R. blastica grown at a dilution rate of 0.05 h^{-1} subjected to analytical protocol as described for Figure 5.3.

centrifugation. However, densitometric scan of the stained protein bands show that the Form I enzyme was synthesised in higher amounts than the Form II. This finding also correlates with the higher activity of the Form I (slower migrating) RuBisCO in the gel slices than the Form II.

When the soluble extract of R. blastica derepressed in RuBisCO (i.e. from cells grown under CO_2-limited in continuous culture) was subjected to partial purification and then analysed by the non-denaturing polyacrylamide gel assay for RuBisCO, only one Form of the enzyme was detected. This was evident from the analysis of the pooled active enzyme fractions from the sucrose gradient centrifugation by non-denaturing polyacrylamide gel assay for RuBisCO enzyme (Figure 5.4). Only one peak of RuBisCO activity was obtained in the gel slices and this peak of activity corresponded to the stained protein band in the gel (Figure 5.4). From the migration pattern of the stained protein, it is evident that it is the Form I enzyme that was synthesised under the CO_2 limited growth conditions.

The sucrose gradient centrifugation procedure however, could not be used to enrich for RuBisCO from the soluble extracts of R. blastica grown under aerobic conditions. Presumeably, this was due to the fact that the enzyme was only synthesised in trace amounts.

Summary of Observations

It has been shown in this study, that under CO_2 limited conditions R. blastica is derepressed for RuBisCO, and only the Form I type is synthesised. It is possible that under CO_2 limitation, this organism needs a highly efficient system to scavenge any available CO_2 in the medium in order to maintain the growth rate at the dilution rate used in

this study. In view of the fact that the Form I enzyme has a low Km for CO_2 (40 μM) as compared with the Form II (Km CO_2 - 104 μM) (see Section 4.5 of this thesis). R. blastica probably synthesises the Form I enzyme which has a higher affinity for CO_2 in order to fix any available CO_2 into cellular carbon so as to maintain the maximal growth rate.

During heterotrophic growth on malate, the two Forms of the RuBisCO enzyme appeared to be synthesised, though at low repressed levels, both at high and limiting light intensities. In each case, the Form I enzyme was present at higher levels than the Form II type. Under these growth conditions, CO_2 was released into the growth medium presumably from the metabolism of malate. The level of CO_2 (up to 3 mM) may be high enough to cause minimal induction of the synthesis of the Form II. These predictions were further investigated (see below) by studying the synthesis of the Form I and Form II enzymes in R. blastica in response to growth of this organism on varying CO_2 concentrations.

5.7 Time Course of the synthesis of ribulose bisphosphate carboxylase/oxygenase in Rhodopseudomonas blastica grown in batch culture on pyruvate-malate medium

The expression of the Form I and the Form II RuBisCO in R. blastica during growth in batch on pyruvate-malate medium was investigated. Figure 5.5 shows the growth curve of R. blastica and the specific activity of the RuBisCO in the soluble extracts during growth. The growth of this organism followed the normal batch culture pattern when cell density was measured as absorbance at 650 nm. The specific activity of RuBisCO in the soluble protein extracts increases steadily

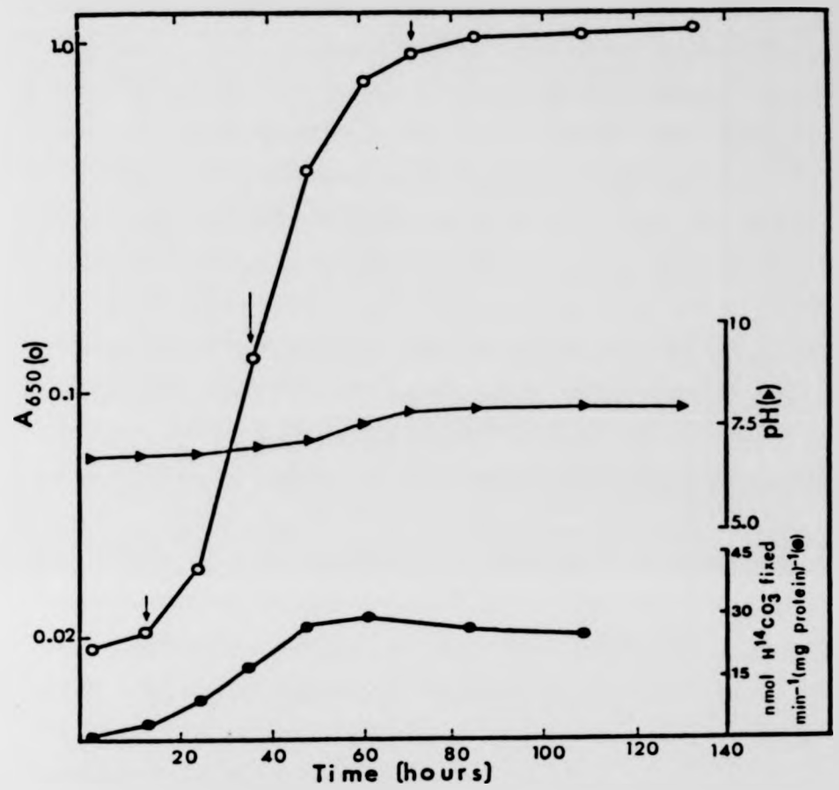


Figure 5.5 Time course of the synthesis of RuBisCO in R. blastica during growth in batch on pyruvate-malate

Carboxylase activity (nmol $H^{14}CO_3$ fixed.min^{-1}.mg protein^{-1}) was determined with soluble protein extracts (●). Arrows indicate times at which cells were harvested and analysed for the presence of one or more different molecular forms of RuBisCO.

during growth, and reached a maximum of 32 nmol min^{-1} mg $protein^{-1}$ at the mid to late exponential phase of growth (Figure 5.5). To determine which of the two forms RuBisCO was synthesised at different phases of growth soluble protein extracts of cells from the early, mid and late exponential phase of growth were subjected to sucrose gradient purification and pooled active fractions subjected to non-denaturing polyacrylamide gel analysis as described before. Clearly, during the early exponential phase of growth, only the Form I enzyme was synthesised as evident from the CO_2 fixing activity profile on sucrose gradient fractions and a single peak of RuBisCO activity in the gel slices which correspond to the stained protein band in the gel (Figure 5.6).

When the soluble extract of cells from the exponential phase of growth was analysed for the Form I and the Form II enzymes the presence of both forms was evident (Figure 5.7). However, during this phase of growth, the Form I enzyme was predominant as shown by its higher activity in the gel slices (Figure 5.7b).

The soluble extract from the early stationary phase, when analysed for RuBisCO, showed a different pattern. During this phase of growth, there was a complete shift from the synthesis of the Form I to that of the Form II enzyme (Figure 5.8). The stained protein bands and their corresponding activity and peaks on densitometric scans show that the Form II enzyme was present in the soluble extracts at a higher level than the Form I. These results therefore, indicate that the two enzymes are differentially expressed during growth of R. blastica in batch culture. It may be that this differential expression is mediated by the level of CO_2 in the growth medium. For instance, during the early exponential

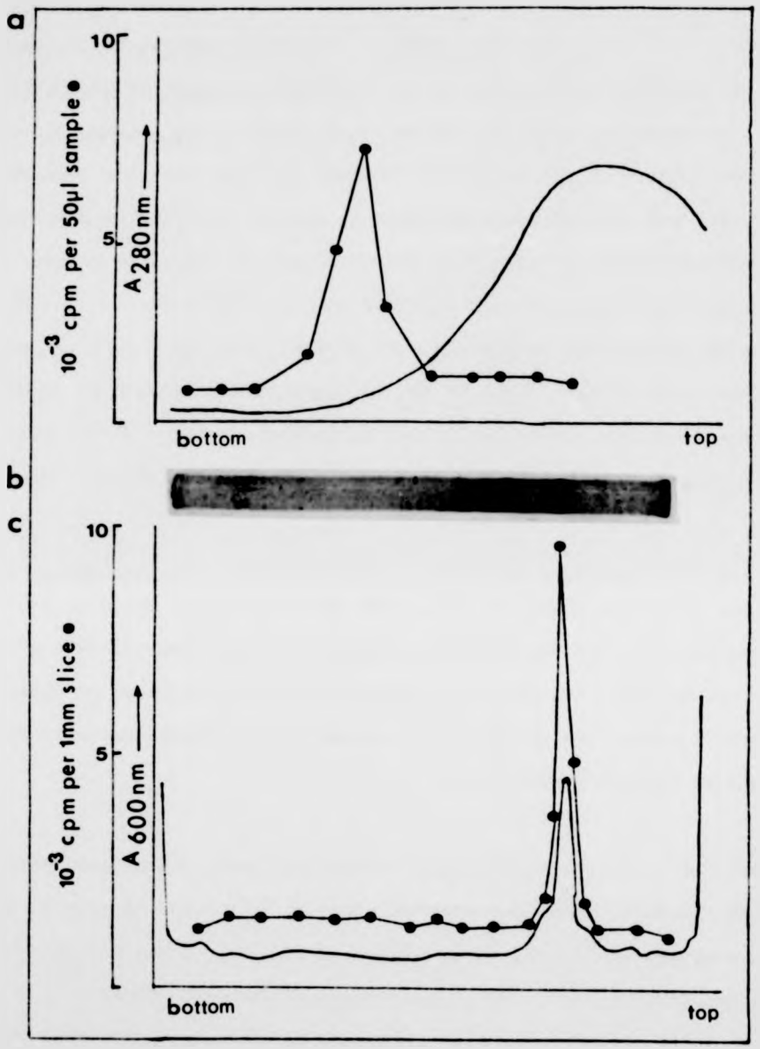


Figure 5.6 Soluble protein extract of R. blastica from the early exponential phase of growth on pyruvate-malate medium centrifuged into a 0.2-0.8 M step sucrose gradient.

a) Enzyme activity is expressed as cpm $^{14}CO_2$ fixed/50 ul sample.

b) A 5% (w/v) non-denaturing polyacrylamide gel electrophoretogram of the pooled active fractions from (a).

c) A duplicate gel from (b) was sliced into 1 mm and each slice assayed for RuBP dependent $^{14}CO_2$ fixing activity.

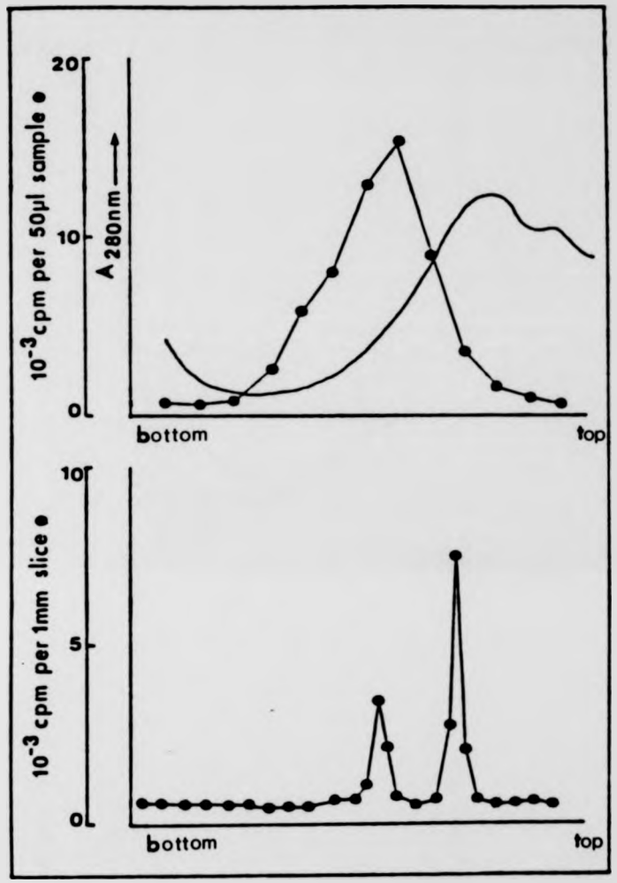


Figure 5.7 Soluble protein extract of B. blastica from mid-exponential phase of growth on pyruvate-malate medium treated as described in Figure 5.6

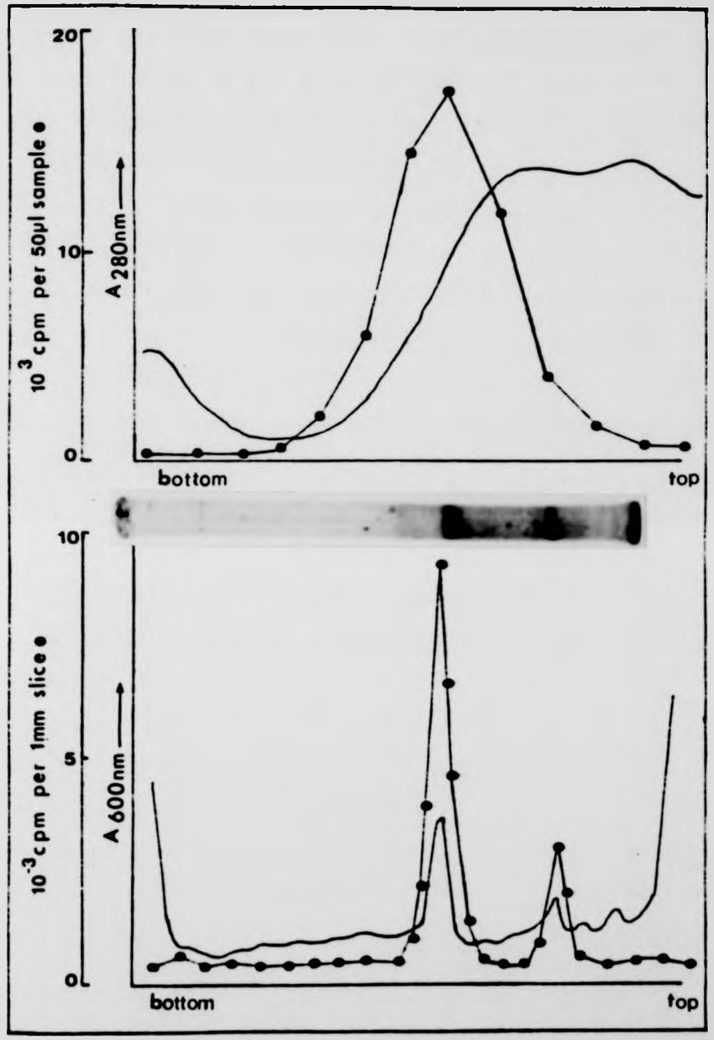


Figure 5.8 Soluble protein extract of R. blastica from the late exponential phase of growth treated as described for Figure 5.6

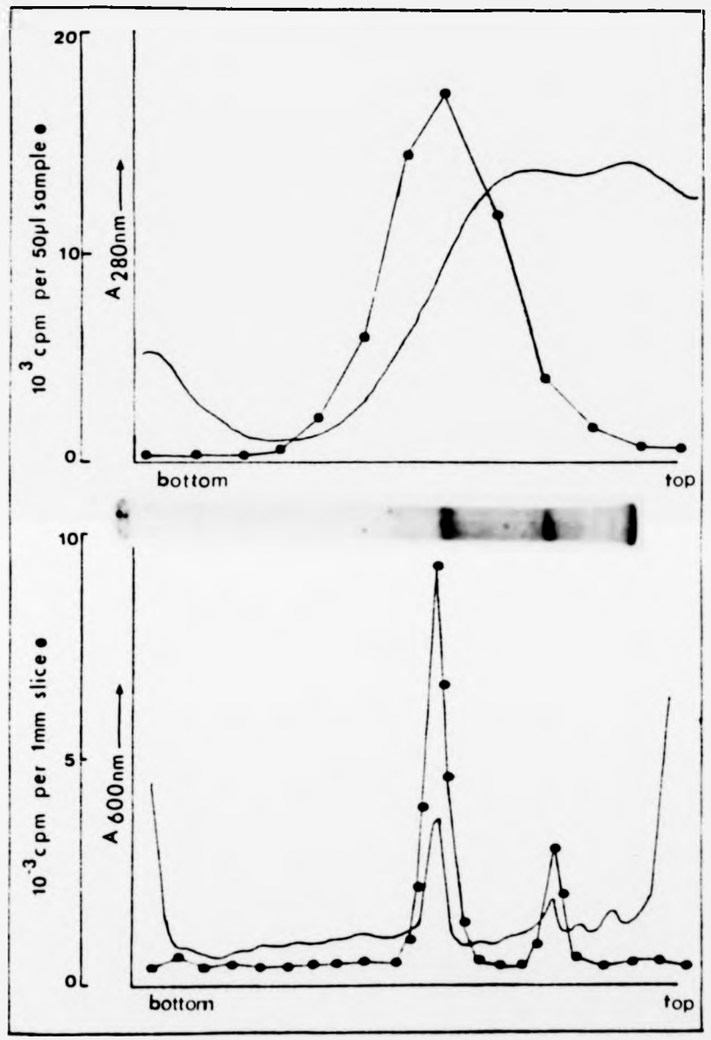


Figure 5.8 Soluble protein extract of R. blastica from the late exponential phase of growth treated as described for Figure 5.6

phase of growth when the level of CO_2 is only in trace amounts the Form I enzyme was the major type synthesised. During the exponential phase of growth, the CO_2 level in the medium was most probably high enough to cause the repression of the Form I enzyme. This repression of the Form I enzyme by high levels of CO_2 may account for its lower level when compared with the Form II RuBisCO at the late log phase, since the preformed Form I enzyme is simply diluted out as growth proceeded to enter stationary phase. The Form II enzyme on the other hand, appeared to be present at mid to late log phase of growth, presumeably as a result of induction by high levels of CO_2 in the growth medium. However, further evidence is needed to substantiate this prediction and this will be reconsidered later.

5.8 Effect of mixed substrates on the levels of ribulose bisphosphate carboxylase/oxygenase in Rhodopseudomonas blastica as a function of growth

Studies with chemostat cultures of R. blastica under different limitations (Section 5.3) showed that this organism synthesised low levels of RuBisCO in the presence of heterotrophic substrates, e.g. malate, but the enzyme was derepressed when supplied with a more reduced electron donor such as butyrate under CO_2 limitation. The effect of added malate to the butyrate-HCO_3 medium on the expression of RuBisCO in R. blastica during growth in batch was investigated (Figure 5.9). On butyrate-bicarbonate medium, high levels of ribulose bisphosphate carboxylase-oxygenase (RuBisCO) were synthesised at the late log phase of growth which was about 70 h in the experiment depicted in Figure 5.9. Addition of malate results in repression of the synthesis of the RuBisCO enzyme in R. blastica during

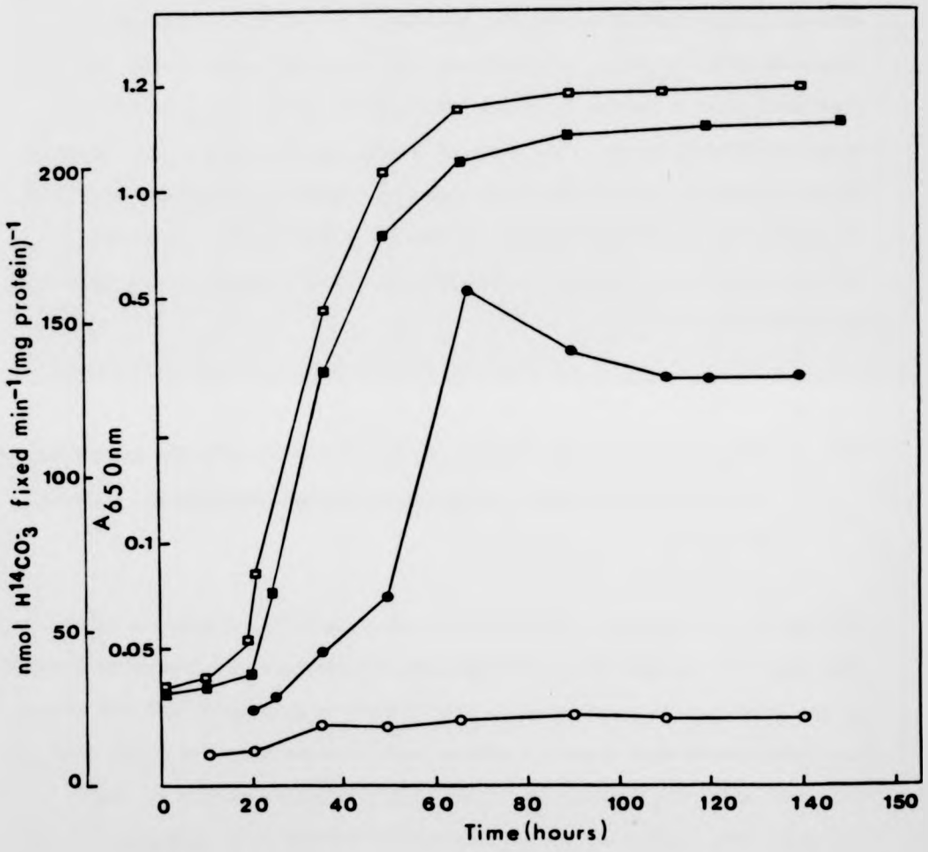


Figure 5.9 The effect of mixed substrates on the growth and the synthesis of ribulose bisphosphate carboxylase-oxygenase in Rhodopseudomonas blastica

A_{650} nm is the absorbance at 650 nm of cells grown on butyrate + malate (□) and on butyrate -bicarbonate (■) medium. Ribulose bisphosphate carboxylase activity (nmol$H^{14}CO_3$ fixed.min^{-1}.mg $protein^{-1}$) was determined with soluble protein extracts of butyrate + malate (○) and butyrate -bicarbonate (●) grown cells.

all growth phases (Figure 5.9). Although the growth rate of R. blastica was the same on butyrate-bicarbonate and on butyrate-malate media, there was a 15-fold increase in RuBisCO activity when this organism was supplied with butyrate as compared with a mixture of butyrate and malate as carbon substrates. The repression of RuBisCO in cells grown on butyrate-malate, may be due to the high levels (about 3 mM when determined as dissolved inorganic carbon) of CO_2 released into the medium presumably from the heterotrophic metabolism of malate. Perhaps during growth of Rhodopseudomonas blastica on a mixture of butyrate and malate, malate is preferentially utilized, thereby avoiding the high energy demanding CO_2 fixation, and consequently the low levels of RuBisCO.

5.9 The in vivo incorporation of [^{35}S] methionine into ribulose bisphosphate carboxylase/oxygenase from Rhodopseudomonas blastica

The synthesis of the Form I and Form II RuBisCO in R. blastica in response to changes in CO_2 concentration in the growth medium was investigated. In these experiments, cells from different phases of growth on butyrate-HCO_3 medium were pulse labelled with [^{35}S] L-methionine and the soluble protein extracts subjected to SDS polyacrylamide slab gel electrophoresis. Gels were subsequently stained for protein and then fluorographed in order to locate the radioactively labelled polypeptides.

Figure 5.10 shows a typical growth curve for Rhodopseudomonas blastica and the levels of ribulose bisphosphate carboxylase-oxygenase in this organism as a function of growth. The growth of R. blastica on the butyrate-bicarbonate medium followed the normal batch growth pattern when

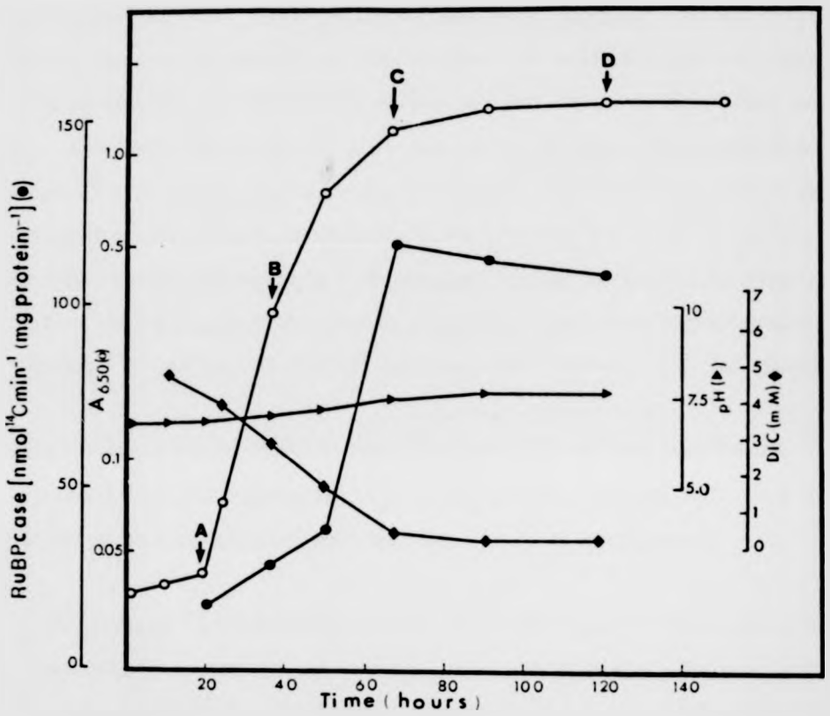


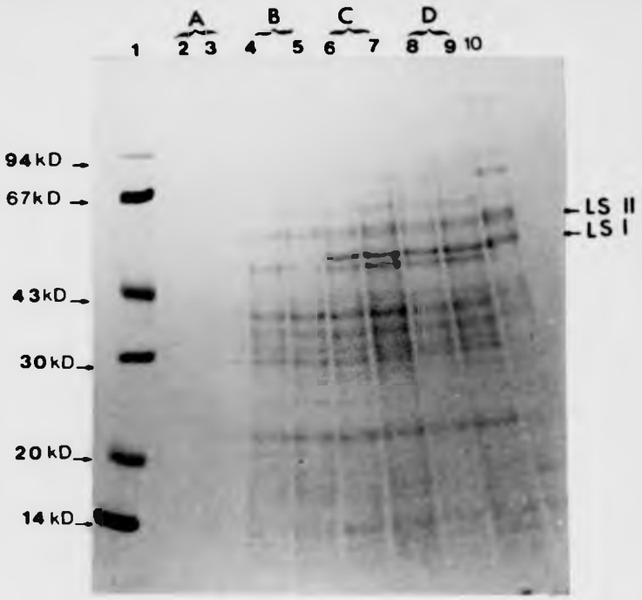
Figure 5.10 Time course of synthesis of ribulose bisphosphate carboxylase-oxygenase in Rhodopseudomonas blastica grown in batch

The mineral salts medium contained 10 mM Na-butyrate and 5 mM sodium bicarbonate (final concentrations). At the times indicated by the arrows (A, B, C, D) samples were removed from the culture and pulse labelled with [^{35}S] L-methionine for 2 min. Soluble protein extracts of the labelled cells were then analysed on SDS polyacrylamide gel.

DIC = dissolved inorganic carbon.

cell density was measured as absorbance at 650nm. However, there appeared to be an inverse correlation between the specific activity of ribulose bisphosphate carboxylase/oxygenase in the soluble protein extract and the level of CO_2 (determined as dissolved inorganic carbon, DIC) in the growth medium (Figure 5.10). During active growth, the CO_2 concentration in the medium decreased sharply, but RuBisCO activity increased steadily, reaching about 30 nmol $^{14}CO_2.min^{-1}.mg\ protein^{-1}$ by the mid log phase. Active growth ceased after the CO_2 concentration in the medium was almost exhausted, but at this point RuBisCO activity increased sharply reaching a maximum of 125 nmol $^{14}CO_2.min^{-1}.mg\ protein^{-1}$ (Figure 5.10). At stationary phase, a decrease in the RuBisCO specific activity was observed, though the amount of the total enzyme unit remained constant, indicating that the enzyme was diluted out - perhaps as a result of synthesis of other proteins of unknown functions.

In order to determine the relative levels of the Form I and Form II RuBisCO synthesised in R. blastica during growth, the soluble protein extracts of [^{35}S] L-methionine labelled cells were subjected to SDS polyacrylamide gel electrophoresis and the gels stained for protein with Coomassie blue and then fluorographed (Figures 5.11 and 5.12). Clearly, the Form II enzyme was present during all phases of growth as is evident from the Coomassie stained polypeptide which co-migrated with the partially purified Form II enzyme from R. blastica (Figure 5.11, lanes 2 to 9). On the other hand, the Form I enzyme appeared to be present in the cell only when it was derepressed for RuBisCO, as evident from the stained polypeptide which co-migrated with the large subunit of the partially purified Form I enzyme (Figure 5.11, lanes 6 to 9). The fluorograph of the stained gel shows substantial differences in [^{35}S] L-methionine incorporation into the Form I and Form II RuBisCO polypeptides



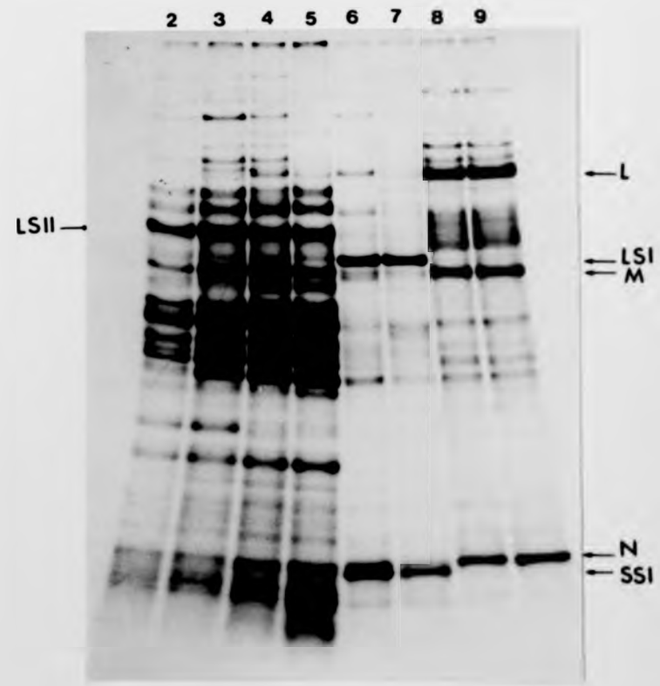
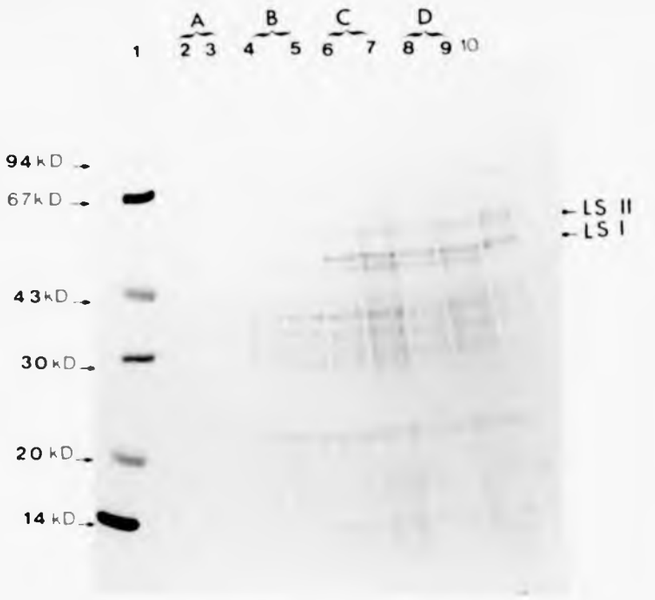


Figure 5.11 Synthesis of ribulose bisphosphate carboxylase-oxygenase in Rhodopseudomonas blastica during growth in batch culture on butyrate- bicarbonate medium

SDS-PAGE (10-30% (w/v) acrylamide) of soluble protein extracts from [^{35}S] L- methionine labelled cells of R. blastica from the experiment shown in Figure 5.10. Lane 1, molecular weight protein standards; lanes 2 and 3, early log phase of growth; lanes 4 and 5, mid log phase; lanes 6 and 7, late log phase; lanes 8 and 9 stationary phase of growth; lane 10, partially purified Form I and Form II RuBisCO (2 µg each) from R. blastica. Even numbered lanes are loaded with equal counts of the soluble protein extract from cells labelled for 2 min with [^{35}S] L-methionine, while odd numbered lanes are loaded with equal counts of 10 min chase with cold methionine. A, B, C and D represent times at which cells were labelled during growth as shown in Figure 5.10. Gels were stained with Coomassie blue.

Figure 5.12 Fluorograph of the gel shown in Figure 5.11

LSI and SSII are respectively, large and small subunits of Form I LSII is the large subunit of the Form II enzyme.



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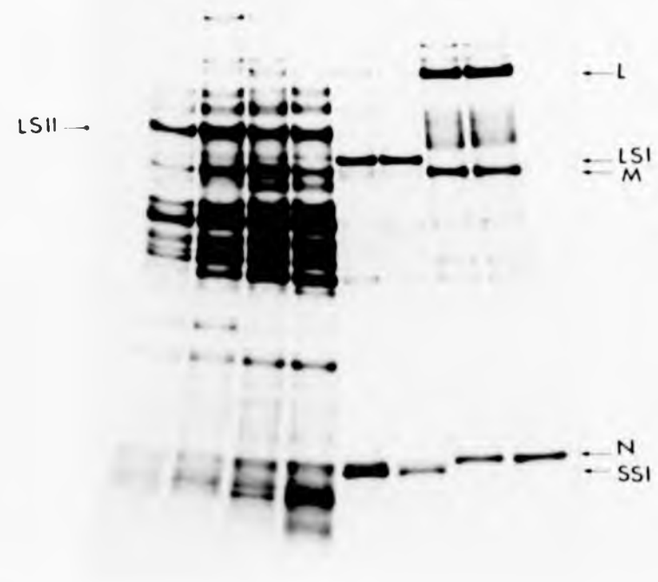


Figure 5.12 Fluorograph of the gel shown in Figure 5.11

LSI and SSII are respectively, large and small subunits of Form I LSII is the large subunit of the Form II enzyme.

(Figure 5.12). During the early and mid exponential phase of growth when the CO_2 concentration in the medium was greater than 1 mM [^{35}S] L-methionine was incorporated only into the large subunit poypeptide of the Form II enzyme, indicating that the Form II enzyme was the major form of the RuBisCO synthesised during these phases of growth. At limiting concentrations of CO_2, i.e. when CO_2 was almost exhausted from the growth medium, and the cells become derepressed for RuBisCO, [^{35}S] L-methionine was incorporated only into the large and small subunits of the Form I enzyme (Figure 5.12, lanes 6 and 7). During this phase, there was no evidence for the incorporation of [^{35}S]-methionine into the Form II enzyme although it was still present in the cell as shown in Figure 5.11. This suggests that at limiting levels of CO_2, in the growth medium, synthesis of the Form II enzyme was switched off and that the increase in RuBisCO specific activity (i.e. from about 30 nmol at log phase to about 125 nmol.min^{-1}.mg protein^{-1} at early stationary phase) was solely due to the switching on and synthesis of the Form I enzyme. When CO_2 was completely exhausted from the medium, i.e. during the stationary phase of growth, the synthesis of the Form I and the Form II enzymes was stopped, as evident from the lack of incorporation of [^{35}S] L-methionine into these proteins (Figure 5.12, lanes 8 and 9). However, there was a significant level of other proteins (L, 68; M, 52; and N, 15 K polypeptides) of unknown function, synthesised during the stationary phase of growth. This may account for the decrease in specific activity of the RuBisCO enzyme in the soluble extracts of cells after the early stationary phase of growth as shown in Figure 5.10.

In order to determine the levels of the Form I and Form II enzymes relative to the total soluble protein synthesised at different phases of growth, stained polypeptides which co-migrated with each of the RuBisCO

subunits were cut out, and counted for radioactivity. Table 5.3 shows the level of Form I and Form II enzymes relative to the total soluble protein synthesised. During the exponential phase of growth, about 0.05% and 0.01% of the total [^{35}S] L-methionine incorporation was in the Form II and the Form I enzyme respectively. During the early stationary phase when the cells were derepressed about 60% of the [^{35}S] L-methionine labelling was incorporated into the Form I enzyme, and there was no incorporation into the Form II enzyme. If the incorporation of [^{35}S] L-methionine is taken as a qualitative measure of the level of protein synthesised, then at CO_2 (bicarbonate) concentation greater than 1 mM, Form I and the Form II enzymes represent about 0.01 and 0.06% respectively, of the total protein synthesised by R. blastica. At limiting levels of CO_2 (i.e. bicarbonate concentrations lower than 0.05 mM), only the Form I enzyme is derepressed, and under this condition, the Form I RuBisCO represents more than 60% of the total soluble protein synthesised. These results therefore, suggest that R. blastica is capable of derepressing only for the Form I enzyme and this occurs only at limiting levels of CO_2. The synthesis of the Form II enzyme on the other hand, is not under this mode of control, i.e. it is only synthesised at low repressed levels under high, perhaps saturating, level of CO_2.

The differential regulation of the synthesis of the Form I and Form II RuBisCO in R. blastica was investigated further by studying the pattern of [^{35}S] L-methionine incorporation into these enzymes in cells which were first grown on limiting levels of CO_2 (i.e. 0.005 mM $NaHCO_3$ + 10 mM butyrate) and then shifted to higher CO_2 concentrations (i.e. 5 mM bicarbonate). In these experiments cells from the different growth conditions were labelled with [^{35}S] L-methionine and soluble protein

Table 5.3 Levels of the Form I and Form II RuBisCO relative to the total soluble protein synthesised in R. blastica at different growth phases in batch on butyrate-bicarbonate medium

Growth Phase	[^{35}S] L-methionine incorporation into Form I and Form II RuBisCO (% of total counts in protein bands from the gel slices)	
	Form I	Form II
A) Early log	0.01	0.06
B) Mid-log	0.01	0.08
C) Late log	60.8	0
D) Stationary phase	0	0.001

extracts were run on SDS polyacrylamide gels. Such gels were stained for protein and then fluorographed in order to locate the radioactively labelled protein.

Figure 5.13 shows the growth pattern of R. blastica under different CO_2 concentrations. Clearly, growth on limiting levels of CO_2 (i.e. 0.005 mM bicarbonate + 10 mM butyrate) was poor, reaching a maximum cell density of 40 mg dry wt.l^{-1}, while cells which were shifted from the low CO_2 to high CO_2 (i.e. 5 mM bicarbonate) medium exhibited a dramatic increase in growth. In the later case, cell density of 308 mg dry wt.l^{-1} of culture was obtained during the exponential phase of growth. These results indicate that the growth of R. blastica on butyrate was dependent on the CO_2 (bicarbonate); perhaps significant amount of cellular carbon was derived from the CO_2 in the medium.

The protein pattern in the soluble extracts of labelled cells, as analysed on SDS polyacrylamide slab gels is shown in Figure 5.14. Clearly, the Form I enzyme was present in detectable levels, in cells grown at low CO_2 (i.e. 0.005 mM bicarbonate) medium and in the cells shifted from the low to high CO_2 (i.e. 5 mM bicarbonate medium). This is evident from the Coomassie stained polypeptide bands which co-migrated with the purified large subunit of the Form I enzyme (Figure 5.14A). The Form II enzyme on the other hand was present in detectable amounts only after the cells were shifted from the low CO_2 medium to one of high CO_2 (i.e. 5 mM bicarbonate) (Figure 5.14A, lanes 5 to 9). The fluorograph of the stained gel is shown in Figure 5.14B. Clearly [^{35}S] L-methionine was incorporated into the large and small subunit of only the Form I enzyme, in cells from the low CO_2 (i.e. 0.005 mM bicarbonate) medium. In fact, radioactivity counts of the solubilized large and small subunits bands of

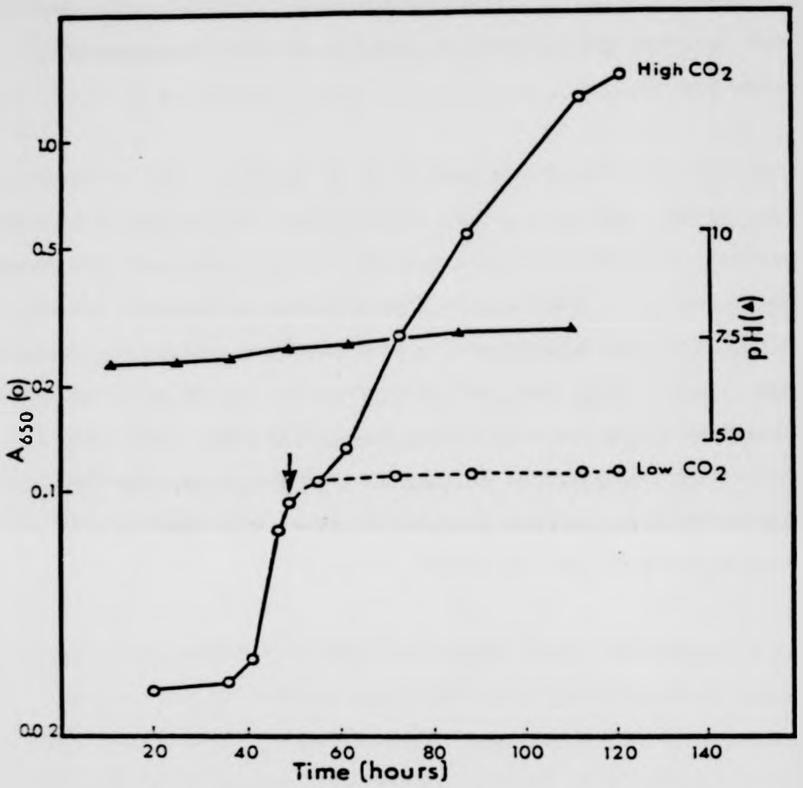
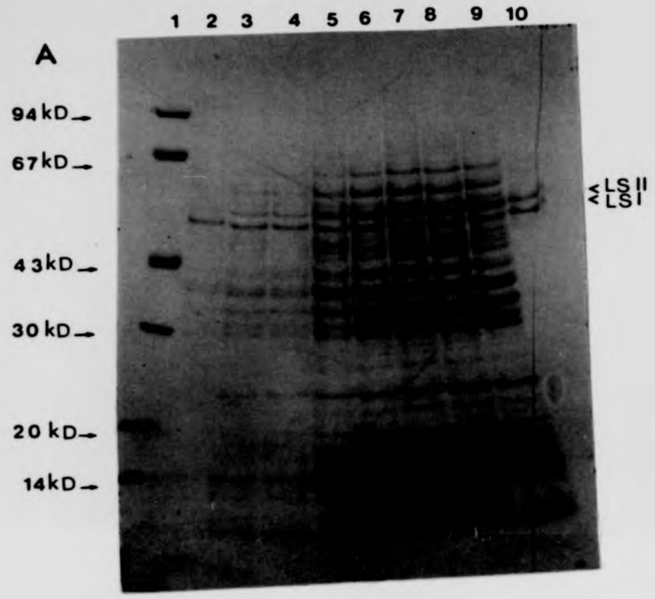


Figure 5.13 Effect of carbon dioxide concentrations (supplied as $NaHCO_3$) on the synthesis of ribulose bisphosphate carboxylase/oxygenase in Rhodopseudomonas blastica

R. blastica was initially grown on mineral salts medium containing 10mM butyrate and low CO_2, i.e. 0.005 mM $NaHCO_3$ (final concentrations). At the time indicated by the arrow, the culture was divided into two halves, one was maintained on the low CO_2 medium (------), and the HCO_3 concentration in the other half increased to 5 mM (high CO_2). At the appropriate time intervals (before and after the shift), cells were removed from the culture and labelled with [^{35}S] L-methionine for 2 min. The soluble protein extracts from the labelled cells were then analysed on SDS polyacrylamide gels (See Figure 5.14).

the Form I enzyme from the gel shows that under the low CO_2 condition, this enzyme represents more than 60% of total soluble protein synthesised (Table 5.4). However, immediately after growing cells were shifted from low to high CO_2 concentration, the synthesis of the Form I enzyme was completely switched off, while the synthesis of the Form II was induced (Figure 5.14b). Although the Form I enzyme could still be detected in the soluble extracts of cells from the high CO_2 medium, as evident from the stained polypeptide bands which co-migrated with the purified Form I enzyme (Figure 5.14a) there was no [^{35}S] L-methionine incorporation into the polypeptides (Figure 5.14b). Qualitative comparison of the radioactivity counts of the solublilized protein bands from the gel shows that the Form II enzyme represents about 0.1% of the total soluble proteins synthesised in R. blastica under the high CO_2 concentrations. It is worth noting that soluble extract of cells which were labelled from the early stationary phase of growth in low CO_2, showed that the protein which co-migrated with the small subunit of the Form I enzyme was being synthesised after the large subunit has been switched off (Figure 5.14b). This may suggest that the synthesis of the large and small subunits of the Form I RuBisCO in Rhodopseudomonas blastica are uncoupled. However, further evidence is needed to substantiate this finding.

The results discussed above indicate that the signal for the repression and derepression of the synthesis of the Form I enzyme is mediated solely by the level of CO_2 in the medium. High levels of CO_2 (i.e. bicarbonate concentration higher than 1 mM) in the medium cause complete repression of the sythesis of the Form I enzyme, while its derepression occurs in CO_2 concentrations less than 1 mM (as bicarbonate). The Form II enzyme is only induced when R. blastica is grown on high CO_2 concentration medium, although under this growth condition, the Form II enzyme is



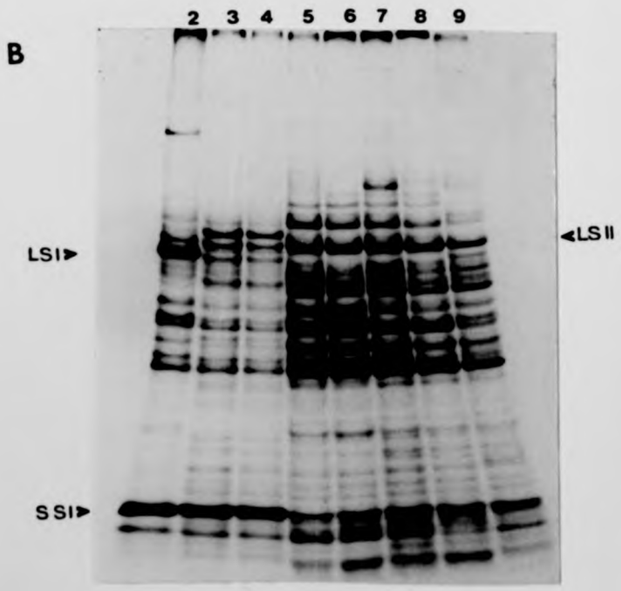
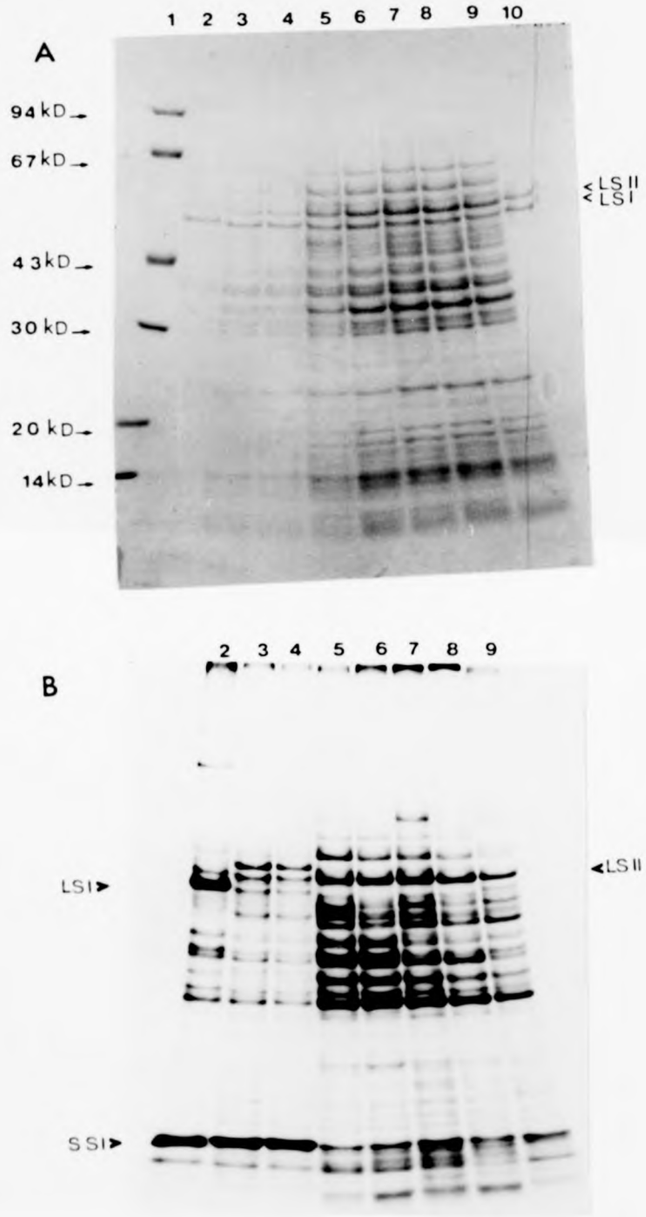


Figure 5.14 Synthesis of ribulose bisphosphate carboxylase-oxygenase in Rhodopseudomonas blastica in response to changes in CO_2 concentration in the growth medium

A) SDS-PAGE (10-30% (w/v) acrylamide) of soluble protein extracts from labelled cells of R. blastica from the experiment depicted in Figure 5.13. Lane 1, molecular weight standards; lane 2, soluble protein exracts from low CO_2 grown cells before the shift to high CO_2; lanes 3 and 4, cells maintained on low CO_2; lanes 5 and 6, 6 h after the shift from low to high CO_2; lanes 7 and 8, 12 h on high CO_2; lane 9, 20 h on high CO_2; lane 10, 2 ug each of purified Form I and Form II RuBisCO from R. blastica. Equal counts were loaded onto the gel, which was stained with Coomassie blue A.R25.

B) Fluorograph of gel A. Lanes 2 to 9 are as described for A. LSI and SSI are large and small subunits of the Form I enzyme respectively. LSII - large subunit of Form II enzyme.



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B) Fluorograph of gel A. Lanes 2 to 9 are as described for A. LSI and SSI are large and small subunits of the Form I enzyme respectively. LSII - large subunit of Form II enzyme.

Table 5.4 Levels of the Form I and Form II RuBisCO relative to the total soluble protein synthesised in R. blastica during growth on low and high levels of CO_2 in the medium

Growth Phase	[^{35}S] L-methionine incorporation into Form I and Form II RuBisCO (% of total counts in protein bands from gel slices)	
	Form I	Form II
Low CO_2 before the shift	62	0.02
Maintained on low CO_2	0 LS, 30 SS?+	0.025
Shift from low to high CO_2		
a) 6 h	0	0.1
b) 12 h	0	0.12

+ LS = large subunit
SS = small subunit

synthesised at low repressed levels.

5.10 General conclusion

It has been shown in this study that photoheterotrophic growth of Rhodopseudomonas blastica on oxidizable organic substrates such as malate reduces the importance of photosynthetic carbon dioxide fixation by the Calvin cycle. Growth of the organisms on more reduced substrates such as butyrate, in the presence of CO_2, results in an increase in the rate of CO_2 fixation. However, it is clear from the results that the rate of photosynthetic CO_2 assimilation by cultures of R. blastica grown photoheterotrophically either on malate or butyrate varies, depending on the stage of growth and method of growth. The variation in the rate is particularly evident when the growth conditions are carefully controlled in continuous cultures.

Two main conditions can be recognised;

1) during growth on CO_2 limited nutrient (as growth on butyrate and bicarbonate in chemostat culture) increasing rates of photosynthetic CO_2 fixation are accompanied by increases in the level of ribulose bisphosphate carboxylase/oxygenase in cell free extracts.

2) During rapid growth in batch and at different dilution rates on malate in continuous culture, the lower levels of the RuBisCO enzyme (compared with growth on butyrate-bicarbonate) are paralleled by a lower rate of carbon dioxide assimilation. This suggests that the changes in the rates

of carbon dioxide fixation are caused by alterations in the degree of represion/or inhibition of the Calvin cycle during heterotrophic growth on these substrates.

Presumably, the low concentration of growth limiting nutrient, i.e. CO_2, reduces the internal concentrations of some metabolites sufficiently to permit the relief of inhibition of activity of enzymes involved in CO_2 fixation (and thus results in high rates of CO_2 assimilation), and the removal of a repressor, thereby resulting in derepression of formation of ribulose bisphosphate carboxylase/oxygenase. On the other hand, the control of photosynthetic carbon dioxide assimilation during photoheterotrophic growth on malate may result from an internal level of malate (or some metabolite derived from malate) effecting the repression of the enzymes concerned in CO_2 assimilation. It may also be possible that this substrate is photoheterotrophically metabolised, thus providing ready intermediates of central metabolism that are assimilated rapidly into major macromolecules such as proteins, the organism thereby avoiding the high energy consuming CO_2 fixation by the Calvin cycle. These may not be sole explanations for the variations in the rate of CO_2 fixation by this organism. For example, Lascelles (1960) has shown that high light intensities reduced the levels of RuBisCO in Rhodopseudomonas sphaeroides and Rhodopseudomonas palustris. Possibly the same effect (though to a lesser extent) is responsible for the reduced rates of CO_2 fixation by R. blastica during growth on malate at high (10,000 lux) as compared with limiting (2,000 lux) light intensities (Table 5.2).

Although derepression of ribulose bisphosphate carboxylase-oxygenase is caused by low limiting levels of CO_2 while repression is by high levels of CO_2 (as bicarbonate) in the growth medium, only the Form I enzyme is

subject to this control mechanism. In all the experiment, the Form II enzyme, when synthesised, was present at low repressed levels and synthesised mainly under high CO_2 concentration. This differential mode of regulation of the synthesis of the two forms of the RuBisCO enzyme, may be due to their differences in the affinity for the substrate, i.e. CO_2. The low molecular weight protein (designated Form II) has a low affinity for CO_2, whereas the high molecular weight enzyme (i.e. Form I) has high affinity for CO_2, comparable to that from green algae and some C_4 plants (Jordan and Ogren, 1981). It would be expected that under low limiting levels of CO_2, Rhodopseudomonas blastica needs a highly efficient system to scavenge any available CO_2 in order to maintain growth rate (since the bulk of the cellular carbon is probably derived from CO_2) - hence the synthesis of the Form I enzyme. At high CO_2 levels, the highly efficient CO_2 fixing system may not be required to maintain the growth rate, hence the synthesis of the low affinity - CO_2 enzyme i.e. the Form II. Perhaps the Form II enzyme was evolved at a time when atmospheric CO_2 was high and O_2 concentration was low. The Form I enzyme was probably evolved to compensate, at least in part, for the geological shift from an atmosphere containing high CO_2 and low oxygen to one of low CO_2 and high oxygen levels, a shift occasioned by the appearance of O_2-evolving photosynthesis (Cloud, 1968). Rhodopseudomonas blastica probably synthesises two different molecular forms of ribulose bisphosphate carboxylase/oxygenase as a means of physiological adaptation to variations in carbon dioxide concentrations in the growth environment.

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